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Antioxidant Capacity of Different Broccoli (*Brassica oleracea*) Genotypes Using the Oxygen Radical Absorbance Capacity (ORAC) Assay

ANNE C. KURILICH,[†] ELIZABETH H. JEFFERY,[†,‡] JOHN A. JUVIK,[§] MATTHEW A. WALLIG,[†,||] AND BARBARA P. KLEIN*[,†,‡]

Division of Nutritional Sciences, Department of Food Science and Human Nutrition, Department of Natural Resources and Environmental Sciences, and Department of Veterinary Pathobiology, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801

Antioxidant capacity of hydrophilic and lipophilic extracts from eight broccoli genotypes was compared using the oxygen radical absorbance capacity (ORAC) assay. Each genotype was analyzed for carotenoid, tocopherol, ascorbic acid, and flavonoid content. Results indicate that the antioxidant capacity of hydrophilic extracts ranged from 65.8 to 121.6 μmol trolox equivalents (TE)/g of tissue, and the capacity of lipophilic extracts ranged from 3.9 to 17.5 μmol TE/g. Ascorbic acid and flavonoid content of the hydrophilic extracts did not explain the total variation in antioxidant capacity of those extracts, suggesting either the presence of other antioxidant components that have yet to be identified or that the known antioxidants are producing synergistic effects. The carotenoids did correlate with antioxidant capacity of the lipophilic extracts and accounted for the majority of the variability in that fraction. The variability in hydrophilic and lipophilic antioxidant capacity found among these genotypes suggests that potential efficacy from antioxidants will vary considerably from genotype to genotype.

KEYWORDS: Broccoli; *Brassica oleracea*; antioxidant; antioxidant capacity; ORAC; carotenoid; tocopherol; ascorbic acid; flavonoid

INTRODUCTION

In recent years, the role of natural dietary antioxidants in disease prevention has been the focus of much investigation. Antioxidants inhibit free radical reactions, and may therefore protect cells against oxidative damage. Several studies indicate that antioxidants found at high levels in fruits and vegetables may aid in the prevention of cancer and cardiovascular disease (*1*−*4*). These antioxidants include fat-soluble vitamins and precursors, such as tocopherols and carotenoids, as well as the water-soluble vitamin ascorbic acid, and flavonoids.

The antioxidant capacity of fruits and vegetables has been determined with a variety of methods. Cao and co-workers (*5*) used the oxygen radical absorbance capacity (ORAC) assay to estimate the antioxidant capacity of 22 vegetables. They found that kale had the highest antioxidant capacity, followed by Brussels sprouts, alfalfa sprouts, beets, spinach, and broccoli, among others. Broccoli (*Brassica oleracea* var. *italica*) also had high antioxidant capacity according to Azuma and colleagues (*6*), who evaluated 25 vegetable extracts using linoleic acid emulsions and phospholipid bilayers. Plumb and co-workers (*7*) employed the 2,2′-azinobis[3-ethylbenzothiazoline-6-sulfonate] (ABTS) method and found that extracts from broccoli and red cabbage were very effective at reducing the ABTS radical, suggesting high antioxidant capacity.

Broccoli naturally contains many antioxidants, including carotenoids, tocopherols, ascorbic acid, and flavonoids (*8*, *9*), and has been reported to have high antioxidant capacity (*5*−*7*, *10*). However, it is not known to what degree the antioxidant capacity varies among broccoli genotypes or to what extent individual antioxidants contribute to the overall antioxidant capacity of the vegetable. Our study was designed to determine the variability in antioxidant capacity among select broccoli genotypes and to see the extent of the relationship between identified antioxidant components and total antioxidant capacity.

MATERIALS AND METHODS

Chemicals. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), which is a hydrophilic analogue of vitamin E; β-phycoerythrin (β-PE) from *Porphydium cruentum*; 2,2′-azobis [2-amidinopropane] dihydrochloride (AAPH); α-tocopherol; γ-tocopherol; L-ascorbic acid; and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO). Lutein, zeaxanthin, β-cryptoxanthin, and β-carotene were purchased from Extrasynthese (Genay, France). HPLC grade hexane, acetonitrile, methanol, and methylene chloride were obtained from Fisher Scientific (Hanover Park, IL).

* To whom correspondence should be addressed at University of Illinois, 268 Bevier Hall, 905 S. Goodwin Ave., Urbana, IL 61801. Phone: 217-333-1325. Fax: 217-333-9368. E-mail: bpklein@uiuc.edu.
[†] Division of Nutritional Sciences.
[‡] Department of Food Science and Human Nutrition.
[§] Department of Natural Resources and Environmental Sciences.
[||] Department of Veterinary Pathobiology.

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Table 1. Broccoli Genotypes Evaluated for Their Antioxidant Capacity

genotype	type	source[a]
Brigadier	commercial hybrid	A
Packman	commercial hybrid	A
Majestic	commercial hybrid	B
Peto #7	inbred	C
Peto #15	hybrid	C
MA 191	inbred	D
EV 6-1	inbred	D
VI 158	doubled haploid	D

[a] Sources: A, USDA Plant Genetic Resource Unit (Cornell University); B, Asgrow Seed Co.; C, Peto Seeds; D, USDA Vegetable Research Center.

Broccoli Genotypes. Freeze-dried powder from three broccoli cultivars (Brigadier, Packman, and Majestic), three inbred lines (Peto #7, Ma 191, and EV 6-1), one experimental hybrid (Peto #15), and one doubled haploid line (VI 158) (**Table 1**) were obtained from the laboratory of Dr. J. A. Juvik at the University of Illinois. These genotypes were grown at the University of Illinois Research Farms in Urbana in either 1996 or 1999. At least three heads from each genotype were harvested at fresh-market maturity and transported on ice to the laboratory where florets were removed from heads, immediately frozen in liquid nitrogen, and stored at −80 °C until lyophilization, at which time they were ground into powder and stored at −20 °C. These genotypes were chosen because our previous studies indicated that they varied widely in their antioxidant component content (*8*).

Sample Preparation. Deionized distilled water (27 mL) was added to freeze-dried broccoli powder (3 g). The slurry was vortexed, centrifuged at 1000*g* for 20 min, and the supernatant was collected. Pellets were extracted twice more with 10−15 mL of water. Pooled extracts from each sample were filtered through Whatman #2 filter paper (Fisher Scientific, Hanover Park, IL) and the filtrate was brought to 60 mL final volume with deionized distilled water (*11*). Aliquots of these hydrophilic extracts were frozen at −20 °C. The pellet was then extracted three times with hexane (50 mL of hexane) to remove the lipophilic compounds. Extracts were pooled, filtered, and adjusted to a final volume of 150 mL. Aliquots of this fraction were evaporated to dryness with a rotary evaporator (Brinkman Instruments Inc, Westbury, NY), and frozen at −20 °C. Dried aliquots of lipophilic extracts represented 1 g of freeze-dried broccoli. All extractions were carried out at room temperature (~22 °C).

ORAC Assay. Samples were assayed according to the following modifications of the procedure reported by Cao and colleagues (*5*). Hydrophilic extracts were thawed and diluted 500× with 75 mM phosphate buffer (pH 7.0). Lipophilic extracts, representing 1 g of freeze-dried broccoli, were reconstituted in 1 mL of DMSO and diluted 100× with 75 mM potassium phosphate buffer/DMSO (93:7 v/v). The assay was carried out in black-walled 96-well plates (Fisher Scientific, Hanover Park, IL). Each well had a final volume of 200 μL. The following reactants were added in order: 25 μL of 75 mM phosphate buffer; either 25 μL of diluted sample, Trolox standard (1 mM final concentration), or blank solution (100 μL of β-PE; 0.76 nM final concentration); and 50 μL of AAPH (41.6 mM final concentration). The blank solution for hydrophilic extracts was 75 mM phosphate buffer, and for lipophilic extracts it was 75 mM phosphate buffer/DMSO (92:8 v/v), so that final DMSO concentration of both samples and blank was 1% of the total volume. Immediately after addition of AAPH, plates were placed in a FLx800 fluorescence plate reader (Bio-Tek Instruments, Inc., Winooski, VT), set with excitation filter 530/25 nm and emission filter 590/35 nm, and then read every 2 min for 2 h to reach a 95% loss of fluorescence. Final fluorescence measurements were expressed relative to the initial reading. Results were calculated based on differences in areas under the β-PE decay curve between the blank and a sample and are expressed as μmol of trolox equivalents (TE)/g dry weight (DW) broccoli tissue (*5*).

Carotenoid and Tocopherol Analysis. Carotenoids and tocopherols were quantified following a modification of our previously published method, so that separation of lutein and zeaxanthin could be achieved (*8*). The modification was as follows: Dried 0.2-g aliquots of lipophilic extracts were reconstituted in 200 μL of DMSO, filtered with 0.45-μm filters, and injected (50 μL) onto a YMC C_{30}, 5 μm, 4.6 × 100 mm column protected by a YMC C_{30} guard column (Waters Chromatography, Milford, MA). Mobile phase was acetonitrile/methanol/methylene chloride (75:20:5 v/v/v), containing 0.05% triethylamine (v/v) and 0.1% butylated hydroxytoluene (v/v), with a flow rate of 1.8 mL/min and a 30 min run time. The HPLC system consisted of a model 510 pump, model 710B autosampler, and model 490E programmable multiwavelength detector (Waters Chromatography, Milford, MA). Carotenoids were monitored at 450 nm and tocopherols were monitored at 290 nm. Lutein, zeaxanthin, β-cryptoxanthin, β-carotene, α-tocopherol, and γ-tocopherol were identified on the basis of retention times of known standards. Standards were prepared as published by Kurilich and Juvik (*12*). Final results were expressed as μmol/g DW broccoli tissue.

Ascorbic Acid Analysis. Aliquots of hydrophilic extracts (50 mg/mL) were thawed, and then prepared by taking 1 mL of extract and adding 0.5 mL of 5% aqueous dithiothreitol and 3.5 mL of 1% aqueous meta-phosphoric acid (*8*). The samples were filtered (0.45 μm) and injected (10 μL) onto a heated (30 °C) Supelcogel C610H column (30 cm × 7.8 mm) that was protected with a Supelcogel C610H guard column (5 cm × 4.6 mm) (Supelco, Inc, Bellefonte, PA). The HPLC system was a HP 1050 series with an autosampler and UV detector set at 210 nm (Hewlett-Packard, Palo Alto, CA). Mobile phase was 0.1% phosphoric acid. Flow rate was 0.5 mL/min and run time was 40 min per sample (*13*). Quantification was obtained using external L-ascorbic acid standards prepared in 5% dithiothreitol and 1% meta-phosphoric acid. Final results were expressed as μmol/g DW broccoli tissue.

Flavonoid Analysis. Aliquots of hydrophilic extracts (50 mg/mL) were thawed and prepared by taking 1 mL of extract and adding 0.6 mL of 6 M hydrochloric acid. Samples were then refluxed at 90 °C for 2 h, cooled to room temperature and filtered (0.45 μm) before injecting 100 μL onto the HPLC *(14)*. The HPLC system consisted of a Waters Alliance 2690 HPLC connected to a model 490E programmable multiwavelength detector (Waters Chromatography, Milford, MA) set at 258 nm. The column was a 5-μm Kingsorb 5 C18, 250 × 4.6 mm (Phenomenex, Torrance, CA) protected by a guard column of the same type. The flow rate was 0.4 mL/min with a gradient of 92% solvent A (water/acetic acid; 99.5:0.5 v/v) and 8% solvent B (acetonitrile/acetic acid; 99.5:0.5 v/v) for 5 min, then linearly to 60% A/40% B over 60 min, held for 5 min, then linearly to 92% A/ 8% B to 66 min and held 4 min to equilibrate (*15*). Identification and quantification were obtained using external standards of quercetin and kaempferol prepared in water/methanol (80:20 v/v). Results were expressed as μmol/g DW broccoli tissue.

Data Analysis. Analysis of variance (*16*) was used to identify significant differences in antioxidant capacity among genotypes. Analysis of variance was also used to identify differences in carotenoid, tocopherol, ascorbic acid, and flavonoid content among the broccoli genotypes; only those genotypes that had measurable levels of the individual antioxidants were used in the analyses. Pearson's correlation coefficients for ORAC results for antioxidant capacity with carotenoids, tocopherols, and ascorbic acid were calculated using means of three sub-samples of bulked tissue (each assayed in triplicate) from each genotype (*16*).

RESULTS

Antioxidant Capacity. Antioxidant capacities of the hydrophilic and lipophilic extracts from eight broccoli genotypes are shown in **Table 2**. For all genotypes, the hydrophilic extracts had much greater antioxidant capacity (80−95% of total) than the lipophilic extracts against reactive oxygen species. MA 191 had the highest ORAC values of the hydrophilic extracts, and the greatest total antioxidant capacity (determined by adding the capacity of the hydrophilic extract and the lipophilic extract). Hydrophilic extracts of cv. Packman had the next highest level of protection against reactive oxygen species followed by EV 6-1, cv. Brigadier, Peto #7, VI 158, cv. Majestic, and Peto #15. Peto #15 was lowest in ORAC values for hydrophilic extracts and total antioxidant capacity.

Table 2. Antioxidant Capacity of Hydrophilic and Lipophilic Broccoli Extracts Measured with the ORAC Assay (μmol TE/g DW Broccoli Tissue)[a]

genotype	hydrophilic extract	lipophilic extract	total[b]
Brigadier	74.6 ± 6.1 BCD	3.9 ± 0.5 D	78.5 ± 6.6 BC
Packman	91.6 ± 5.9 B	6.6 ± 1.8 CD	98.2 ± 7.7 B
Majestic	51.2 ± 1.5 DE	0.5 ± 0.2 D	51.7 ± 1.7 CD
Peto #7	71.1 ± 0.3 BCD	1.7 ± 0.3 D	72.7 ± 0.6 BC
Peto #15	38.1 ± 2.4 E	3.9 ± 0.3 DE	42.0 ± 2.7 D
MA 191	121.6 ± 18.7 A	15.2 ± 4.5 AB	136.8 ± 23.2 A
EV 6-1	78.8 ± 27.4 BC	3.0 ± 0.5 DE	81.8 ± 27.9 B
VI 158	65.8 ± 4.4 CD	17.5 ± 0.7 A	83.3 ± 5.1 B

[a] Mean ± SD, $n = 3$. Values with the same letter are not significantly different ($P < 0.05$). [b] Total derived from adding value from the hydrophilic extract to that of the lipophilic extract.

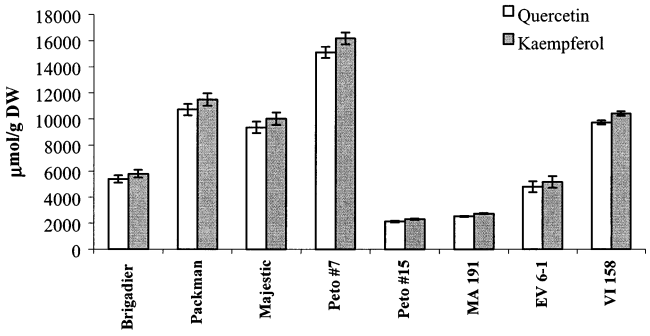

Figure 1. Quercetin and kaempferol contents of hydrophilic broccoli extracts.

Although the antioxidant capacity of the lipophilic extracts was far less than that of the hydrophilic extracts, differences among genotypes were observed. VI 158 and MA 191 had significantly greater antioxidant capacity compared to all other genotypes (**Table 2**).

Content of Individual Antioxidant Components in Extracts. No significant differences were observed in ascorbic acid content (range = 43.5−46.5 μmol/g, $p = 0.31$) of hydrophilic extracts among the genotypes. However, there were significant differences in the flavonoid compounds quercetin ($p < 0.0001$) and kaempferol ($p < 0.0001$) in the hydrophilic extracts from the eight different broccoli genotypes (**Figure 1**). Peto #7 had significantly more of both components compared to other genotypes, whereas MA 191 and Peto #15 had the least amount of both components. Very similar amounts of quercetin and kaempferol were found within each genotype, but in every case kaempferol levels were slightly higher.

Contents of lutein, zeaxanthin, α-tocopherol, and γ-tocopherol are shown in **Figure 2**. Significant differences were observed between genotypes for lutein ($p < 0.0002$), zeaxanthin ($p < 0.0032$), and α-tocopherol ($p < 0.0080$), but not for γ-tocopherol ($p = 0.76$). Lutein was detected in lipophilic extracts of cv. Packman, Peto #15, MA 191, EV 6-1, and VI 158, but not in those of cv. Brigadier, cv. Majestic, or Peto #7. Zeaxanthin was detected only in cv. Packman and VI 158. Cultivar Packman, cv. Majestic, Peto #7, MA 191, and VI 158 contained measurable levels of α-tocopherol. Cultivars Brigadier, Packman, and Majestic, and VI 158 contained measurable levels of γ-tocopherol.

Correlation of Antioxidant Capacity with Individual Antioxidant Components. Antioxidant capacity of the hydrophilic and lipophilic broccoli extracts was correlated with individual antioxidant components using data from the means

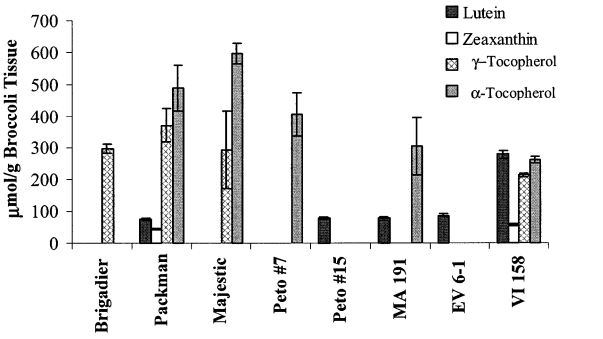

Figure 2. Carotenoid and tocopherol contents of lipophilic broccoli extracts.

of two replicate measurements of the individual antioxidant components and Pearson's correlation coefficients. Results indicated that ascorbic acid ($r = 0.31$, $p = 0.08$), quercetin ($r = 0.36$, $p = 0.10$), and kaempferol ($r = 0.36$, $p = 0.10$) were not correlated with antioxidant capacity of the hydrophilic extracts, and that antioxidant capacity of lipophilic extracts was correlated with lutein ($r = 0.57$, $p = 0.05$, $R^2 = 0.32$) and zeaxanthin ($r = 0.81$, $p = 0.02$, $R^2 = 0.65$), but not with α-tocopherol ($r = 0.37$, $p = 0.16$) or γ-tocopherol ($r = -0.17$, $p = 0.53$).

DISCUSSION

Broccoli is a vegetable that is thought to contain a high level of antioxidant activity (*5*, *7*, *10*). However, until now, no study had considered the degree of variability in antioxidant capacity that exists among broccoli genotypes (**Table 2**). Our results from this study indicate that antioxidant capacity, measured by the ORAC assay of both hydrophilic and lipophilic extracts of broccoli, differs among genotypes. The total antioxidant capacity (hydrophilic + lipophilic) of MA 191 (136.8 μmol TE/g DW), which had the highest activity, was 3-fold greater than that of the genotype with the lowest activity, Peto #15 (42.0 μmol TE/g DW). Cao and co-workers (*5*) used the ORAC assay to determine the antioxidant capacity of 22 vegetables and found that broccoli (unknown genotype) had a capacity of 59 μmol TE/g DW, which falls in the middle of the range found in this study.

Although both hydrophilic and lipophilic extracts demonstrated antioxidant capacity, the hydrophilic extracts were responsible for 80 to 95% of the total antioxidant capacity using the ORAC assay. It is perhaps not surprising that the lipophilic extracts produced little antioxidant capacity, because the ORAC assay is an aqueous system with an aqueous control (Trolox), radical generator, and fluorescent probe. Therefore, it may not accurately reflect the antioxidant capacity of nonpolar components. Recently, an antioxidant capacity assay that uses a lipophilic radical generator and fluorescent probe (*17*) was published and might be a more appropriate system for analysis of lipid-containing extracts. Aldini and co-workers compared the rate of β-carotene depletion under hydrophilic and lipophilic conditions and found that using AAPH in an aqueous environment had little effect on β-carotene oxidation, but β-carotene was rapidly oxidized by the lipophilic radical generator 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile)(*17*). These findings indicate that our antioxidant data concerning the lipophilic extracts should be used with caution.

Several investigators suggested that the antioxidant compounds found at high levels in broccoli and other vegetables and fruits are responsible for their antioxidant capacity (*18*−*20*). Emmons et al. (*21*) reported that tocopherol content of oats

was correlated with ORAC activity of the oat extracts. Azuma et al. (*10*) indicated that the ascorbic acid and total polyphenol content of vegetables was correlated with antioxidant capacity in linoleic acid emulsions and phospholipid bilayers. Ehlenfeldt and Prior (*22*) also found that phenolic content of blueberries was correlated with their ORAC values. However, in our present study, few of the individual antioxidants analyzed were correlated with the antioxidant capacity of the extracts. Ascorbic acid content of hydrophilic extracts did not differ among the genotypes and therefore no correlation was expected. This finding is similar to that reported by Wang et al. (*23*) who showed that the antioxidant capacity of several fruits could not be accounted for by their ascorbic acid content.

Quercetin and kaempferol contents also did not correlate well with antioxidant capacity, suggesting that either there are other antioxidant components yet to be identified in the hydrophilic extracts, or possibly that a combination of individual antioxidants are producing synergistic effects. Among the genotypes analyzed in this study, quercetin content ranged from ~2141 μmol/g DW in Peto #15 to ~15 123 μmol/g DW in Peto #7, whereas kaempferol content ranged from ~2328 μmol/g DW in Peto #15 to ~16 181 μmol/g DW in Peto #7. For comparison with data from other investigators, on a fresh weight (FW) basis, these ranges would be: quercetin = 1.1−7.5 μg/g FW, and kaempferol = 1.2−8.5 μg/g FW. This conversion was based on our previous studies, which indicated that broccoli is composed of 85% moisture and 15% dry matter (*8*). These values are much lower than those reported by Plumb et al. (*9*) who found the quercetin and kaempferol glycoside content of broccoli florets to be 65 and 166 μg/g FW. Justesen and coworkers (*14*) reported that broccoli contained about 70 μg/g FW of quercetin and 100 μg/g FW of kaempferol. The differences noted in flavonoid levels between other studies and this one could be due to the extraction method used. Studies specifically measuring flavonoid content typically extract flavonoids in water/methanol (50:50, v/v) (*24*). However, studies reporting antioxidant capacity of vegetables or fruit typically use, as we did, a water extract that may not be selective enough to obtain high recovery of flavonoids (*5*). Unfortunately, recovery studies were not carried out.

Lutein and zeaxanthin contents of the lipophilic extracts did correlate with antioxidant capacity, suggesting that these carotenoids play a role in the overall antioxidant capacity. Variation in lutein content of the extracts accounted for 32% of the total variation of antioxidant capacity, while variation in zeaxanthin content was responsible for 65% of the variation. As with ascorbic acid, γ-tocopherol content did not vary among genotypes, and, therefore, no correlations were expected and none were found. α-Tocopherol content of the extracts did vary significantly, but no correlation was observed between α-tocopherol content and antioxidant capacity. In the ORAC assay, it appears that α-tocopherol does not contribute substantially to the antioxidant capacity of the lipid-containing extracts.

The present results indicate that broccoli extracts are protective against reactive oxygen species, but there is variability in the level of protection among genotypes. Ascorbic acid, quercetin, and kaempferol content of the hydrophilic extracts cannot explain the variability in antioxidant capacity of those extracts. Lutein and zeaxanthin were correlated with antioxidant capacity of the lipophilic extracts and explain most of the variability found within that fraction. Differences in antioxidant capacity found among the genotypes tested indicates that efficacy from antioxidants may vary from genotype to genotype. Unless breeders, growers, and, ultimately, consumers are aware of this variability, they may not select the varieties with the most potential for health benefits.

ABBREVIATIONS USED

ABTS, 2,2′-azinobis[3-ethylbenzothiazoline-6-sulfonate]; AAPH, 2,2′-azobis[2-amidinopropane] dihydrochloride; β-PE, β-phycoerythrin; DMSO, dimethyl sulfoxide; DW, dry weight; FW, fresh weight; HPLC, high-performance liquid chromatography; ORAC, oxygen radical absorbance capacity; TE, trolox equivalents.

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