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# Food Matrix Effects on Bioactivity of Broccoli-Derived Sulforaphane in Liver and Colon of F344 Rats

ANNA-SIGRID KECK,† QINGYAN QIAO,† AND ELIZABETH H. JEFFERY*,†,§

Division of Nutritional Sciences and Department of Food Science and Human Nutrition, University of Illinois, Urbana, Illinois 61801

Sulforaphane (SF) is considered to be the major anticarcinogenic component in broccoli. The effects of feeding rats purified SF (5 mmol/kg of diet), broccoli containing SF formed in situ during laboratory hydrolysis (broccoli-HP; 20% freeze-dried broccoli diet, 0.16 mmol of SF/kg of diet), and broccoli containing intact glucosinolates (broccoli-GS; 20% freeze-dried broccoli diet, 2.2 mmol of glucoraphanin/kg of diet) were compared. Rats (male F344 rats, five per group) were fed control (modified AIN-76 B-40), SF, broccoli-HP, or broccoli-GS for 5 days. In rats fed broccoli-GS, quinone reductase activities (QR) in the colon and liver were greater (4.5- and 1.4-fold over control, respectively) than in rats fed broccoli-HP (3.2- and 1.1-fold over control, respectively). Broccoli-GS and SF diets increased QR to the same extent, even though the broccoli-GS diet contained far less SF (as the unhydrolyzed glucosinolate, glucoraphanin) than the purified SF diet. In a second experiment, rats were fed one of six diets for 5 days: (1) control; (2) 20% broccoli-GS; (3) diet 2 + low SF (0.16 mmol/kg of diet); (4) diet 2 + high SF (5 mmol/kg of diet); (5) low SF (0.32 mmol/kg of diet); or (6) high SF (5.16 mmol/kg of diet). In both liver and colon, QR was increased most by high SF plus broccoli-GS; individually, high SF and broccoli-GS had similar effects, and adding the low-dose SF to broccoli-GS had either no effect or a negative effect. In both experiments, urinary SF-mercapturic acid correlated with QR activity, not with dietary intake. It was concluded that all diets were substantially more effective in the colon than in the liver and that broccoli-GS was more potent than SF or broccoli-HP.

**KEYWORDS: Sulforaphane; broccoli; mercapturic acid; urinary excretion; detoxification enzymes**

## INTRODUCTION

Diet is thought to be a factor in 30−70% of all cancer cases (*1*). Consumption of a diet high in fruits and vegetables has been shown to decrease the risk for a number of cancers (*2*), and broccoli has been shown to be protective in both epidemiological studies (*3*) and animal studies (*4*). Broccoli is unique among the common cruciferous vegetables in that it contains high levels of the aliphatic glucosinolate glucoraphanin (*5*). Upon hydrolysis, glucoraphanin produces several products including the bioactive isothiocyanate sulforaphane (SF) (*6*). Not only do glucoraphanin concentrations differ considerably across broccoli varieties [0.8−21.7 $\mu$mol of glucoraphanin/g of dry weight (DW)] (*5*), but the extent to which they form SF as the hydrolysis product may also vary (*7*). When the plant is chopped, crushed, or chewed, glucoraphanin and other glucosinolates are hydrolyzed by the endogenous enzyme, myrosinase (*8*). If myrosinase is not available to hydrolyze the glucosinolates (e.g., in cooked whole vegetables), microflora in the lower gut may also hydrolyze the thioglucoside linkage (*9*, *10*). Therefore, the percentage of SF in a broccoli meal may vary depending upon conditions of hydrolysis, food handling, and preparation procedures. If research on the healthful effects of broccoli and broccoli components is to be of use to the public, we need to determine how to prepare broccoli and broccoli-derived supplements for greatest efficacy.

In animal studies, dietary freeze-dried broccoli has been found to offer protection against several cancers (*11*). Furthermore, broccoli-derived SF has been shown to protect against azoxymethane-induced colon cancer and dimethyl benzanthracene-induced mammary cancer (*12*, *13*) and to up-regulate phase II detoxification enzymes, such as quinone reductase (QR) and glutathione *S*-transferases (GST) (*14*, *15*). Up-regulation of phase II detoxification enzymes is thought to be a biomarker for anticarcinogenesis (*16*). In cell culture, low levels of purified SF (0.5−1 $\mu$M) up-regulate phase II detoxification enzymes and inhibit certain phase I cytochrome P450 enzymes (*14*, *17*, *18*). Several studies have evaluated the effect of SF on detoxification enzymes in animals. Mice administered 15−17 $\mu$mol of SF [~500 $\mu$mol/kg of body weight (BW)] daily for 5 days elicited a 2.5-fold increase in hepatic QR activity and a 2.6-fold increase in mammary QR activity (*14*, *15*). When male F344 rats were given SF daily by gavage for 5 days, 500 or 1000, but not 200,

* Address correspondence to this author at 499 Bevier Hall, The University of Illinois at Champaign−Urbana, 905 S. Goodwin Ave., Urbana, IL 61801 [telephone (217) 333-3820; fax (217) 265-0925; e-mail ejeffery@uiuc.edu].

† Division of Nutritional Sciences.

§ Department of Food Science and Human Nutrition.

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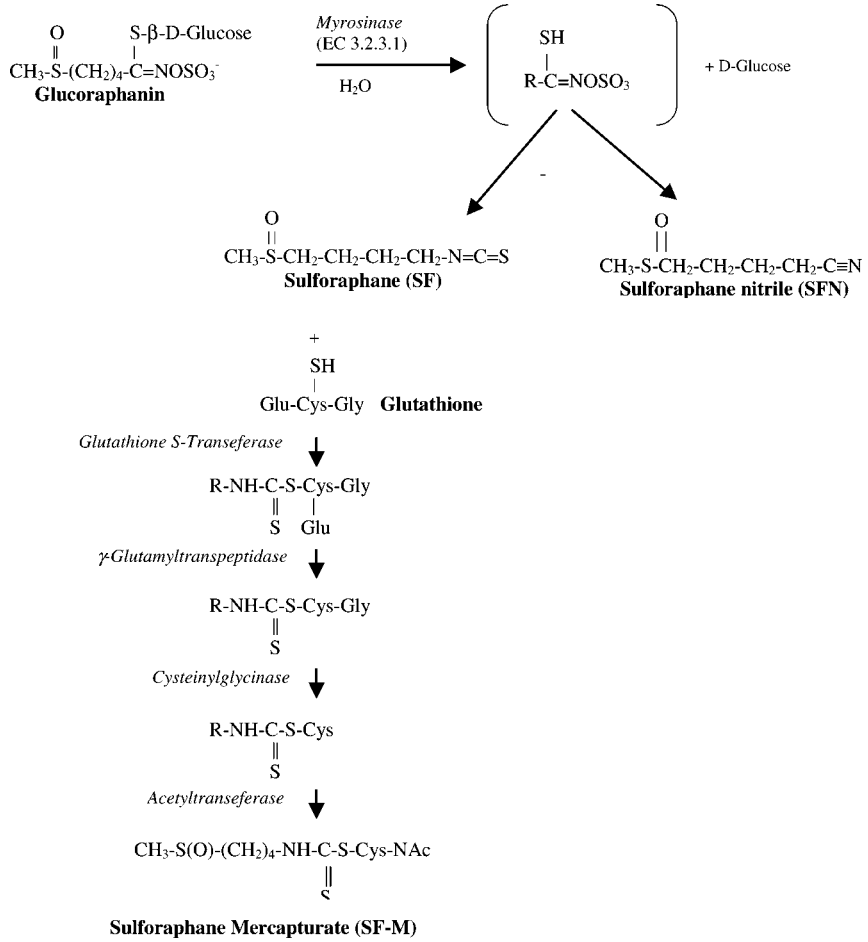


**Figure 1.** Conversion of glucoraphanin to sulforaphane (SF) by the endogenous broccoli enzyme, myrosinase. After digestion, SF is further metabolized by sequential enzymatic reactions to sulforaphane mercapturic acid (SF-M). The R group is $CH_3SO(CH_2)_4$–.

$\mu$mol of SF/kg of BW caused significant increases in hepatic, pancreatic, and colonic QR and GST activities (*18*). Yet freeze-dried broccoli, added to the rodent diet at 10 or 20% and providing <200 $\mu$mol of SF/kg, has consistently been reported to up-regulate phase II detoxification enzymes such as GST and QR (*4*, *19*).

Bioavailability, efficacy, and potency of glucosinolate hydrolysis products have not been addressed in detail. In humans, broccoli feeding trials have evaluated efficacy by measuring plasma GST levels (*20*, *21*), whereas others have determined changes in detoxification enzyme levels indirectly by measuring changes in excretion of metabolites of various compounds, such as caffeine (*22*). Urinary excretion of total isothiocyanates or the specific mercapturic acid conjugates of allyl isothiocyanate (AITC) have been used as relative measures of isothiocyanate dose following a cruciferous vegetable meal in rats and humans (*23*, *24*). More specifically, broccoli and broccoli sprouts meals have been found to increase urinary excretion of total isothiocyanates (*13*, *25*). Isolated SF administered intraperitoneally (ip) to rats has been shown to have a dose relationship to SF mercapturic acid (SF-M) excretion (*26*), with 60% of the dose excreted as SF-M. There are no studies of SF-M excretion following oral SF, in rats or humans. However, there is a clinical study, reporting SF-M excretion following dietary broccoli (*27*); that study found <12% of calculated dietary SF excreted as SF-M and did not estimate bioactivity (*27*). The conversion of glucoraphanin to SF mediated by myrosinase and the metabolism of SF to its mercapturic acid conjugate are illustrated in **Figure 1**.

The objectives of this study were (I) to compare hepatic up-regulation of QR in rats fed diets containing broccoli prehydrolyzed to release SF from glucoraphanin (broccoli-HP) and broccoli containing intact GS (broccoli-GS) and purified SF, in an effort to determine if the broccoli matrix facilitates or aggravates uptake of SF; (II) to compare SF-M excretion from dietary broccoli and oral SF; and (III) to determine if the up-regulation of QR activity in colon and/or liver correlates with urinary SF-M following both broccoli and SF ingestion.

## MATERIALS AND METHODS

**Materials.** Solvents used for extraction and purification were of reagent grade, and solvents used for GC or HPLC analysis were of HPLC grade. All solvents were purchased from Fisher Scientific (Fair Lawn, NJ). Unless otherwise noted, all other materials used in this study were obtained from Sigma (St. Louis, MO). Sulforaphane was purified according to the method of Matusheski and co-workers (*7*); purity was determined to be >99% by GC analysis.

**Broccoli Cultivation.** Broccoli (*Brassica oleracea* var. Italica cv. Majestic) was grown at the University of Illinois, Urbana, IL. At harvest, heads were cut from the mature plants and held on ice for <4 h. Florets (2 in. long) were cut, plunged into liquid nitrogen, and stored at −80 °C until freeze-dried. The freeze-dried broccoli samples were finely ground in a water-cooled analytic mill and stored at −20 °C until used.

**Diet Preparations.** A modified AIN-76 B-40 diet was used as the control diet. The caloric value of the experimental diets was equalized to that of the control diet, and fiber content was adjusted to that of the 20% broccoli diet. The nutritive values for broccoli were obtained from the USDA Nutrient Database for standard reference, release 13 (November 1999). In the purified SF diets, the SF was premixed with

**Table 1.** Formulations of the Modified AIN-76 B-40 Control Diet and 20% Broccoli Test Diets Used in This Study[a]

| ingredient | control diet (%) | 20% broccoli diet (%) |
|---|---|---|
| corn starch | 38.7 | 36.9 |
| sucrose | 25.0 | 22.0 |
| casein, purified, high-nitrogen | 20.0 | 13.7 |
| alphacel, non-nutritive bulk | 6.3 | 0.0 |
| broccoli | 0.0 | 20.0 |
| DL-methionine | 0.3 | 0.3 |
| choline bitartrate | 0.2 | 0.2 |
| AIN mineral mix | 3.5 | 1.6 |
| AIN vitamin mix | 1.0 | 1.0 |
| corn oil | 5.0 | 4.3 |
| total | 100 | 100 |

[a] Nutritional value information of the control diet was retrieved from ICN Biochemicals (Cleveland, OH), and the nutritive value for broccoli was obtained from the USDA Nutrient Database for Standard Reference, release 13 (Nov 1999).

corn oil for 15 min before being mixed with the rest of the diet. All experimental diets (**Table 1**) were stored in the dark for fewer than 10 days at 4 °C in airtight plastic bags. A previous study showed that diets containing isothiocyanates may be stored for at least 2 weeks without loss of activity (*28*). Hydrolyzed broccoli (broccoli-HP) was prepared as follows: one part of freeze-dried broccoli was mixed with nine parts of deionized water for 24 h at room temperature in the dark to allow the endogenous myrosinase to hydrolyze intact glucosinolates to bioactive glucosinolate hydrolysis products. The broccoli slurry was frozen and freeze-dried.

**Characterization of Powdered Broccoli Preparations.** The glucoraphanin content of broccoli-GS (11.0 μmol of glucoraphanin/g of DW) was determined by HPLC following the method of Kushad et al. (*5*). Sulforaphane content of the freeze-dried broccoli-HP (0.8 ± 0.1 μmol/g of DW) was estimated according to the method of Matusheski et al. (*7*). When the two powdered preparations, broccoli-HP and broccoli-GS, were subjected to hydrolysis with 90% water for 24 h, there was no difference in SF recovery between broccoli-HP (0.8 ± 0.2 μmol/g of DW) and broccoli-GS (0.8 ± 0.1 μmol/g of DW), showing that freeze-drying the broccoli-HP sample had not caused loss of SF. Furthermore, there was no loss of SF between broccoli samples before or after exposure to air at room temperature for 24 h, simulating the exposure of the diet to air during the feeding period. The finding of no loss of SF during storage was in agreement with Stoner and co-workers (*28*). In agreement with Matusheski et al. (*7*), the major hydrolysis product of glucoraphanin was SF nitrile (13.1 ± 1.9 μmol/g of DW in rehydrolyzed broccoli-HP and 11.8 ± 0.3 μmol/g of DW in hydrolyzed broccoli-GS), and only ~6% of the glucoraphanin formed SF.

**Animals and Housing.** Fisher 344 rats (weanling, males 40−60 g, Harlan, Indianapolis, IN) were housed individually, in shoebox cages with corncob bedding, in a room with a controlled environment with a 12 h light/dark cycle under uniform temperature and humidity. Animal use was approved by the Animal Care and Use Committee of the University of Illinois. Animals were acclimated over 6 days; during the first 2 days, they received rat chow pellets followed by at least 4 days of AIN-76 B-40 powdered control diet (ICN Biochemicals, Cleveland, OH), modified as shown in **Table 1**. During the acclimation period, food was available for 24 h and provided fresh daily. The animals received water ad libitum throughout the study.

**Experimental Design.** In experiment 1 rats were fed one of four different diets during the 5-day experimental period: (1) control (modified AIN-76 B-40) that provided no SF or glucoraphanin; (2) 20% broccoli-HP (containing 0.16 mmol of SF/kg of diet that provided ~1.6 μmol of SF/rat/day); (3) 20% broccoli-GS (containing 2.2 mmol of glucoraphanin/kg of diet that provided ~22 μmol of glucoraphanin/rat/day); or (4) purified SF (5.0 mmol of SF/kg of diet that provided ~50 μmol of SF/rat/day). Doses and duration of feeding were chosen on the basis of previous studies from this laboratory (*18*, *29*). In experiment 2 rats were fed one of six different diets during the 5-day experimental period: (1) control diet (modified AIN-76 B-40) that provided no SF or glucoraphanin; (2) 20% broccoli-GS containing 2.2 mmol of glucoraphanin/kg of diet that provided ~22 μmol of glucoraphanin/rat/day; (3) 20% broccoli-GS + 0.16 mmol SF/kg of diet that provided ~22 μmol of glucoraphanin and 1.6 μmol of SF/rat/day; (4) 0.32 mmol of SF/kg of diet that provided ~3.2 μmol of SF/rat/day; (5) 20% broccoli-GS + 5 mmol SF/kg of diet that provided ~22 μmol of glucoraphanin and 50 μmol of SF/rat/day; or (6) 5.16 mmol of SF/kg of diet that provided ~51.6 μmol of SF/rat/day. The levels of SF added to the diets were chosen to reflect the amount present in the broccoli-HP diet from experiment 1 (0.16 mmol of SF/kg of diet) added either to a broccoli-GS diet or to a high-SF diet. In both experiments, food was available for 24 h and provided fresh daily during the 5-day experimental period. On the last day (day 5) of experiment 1 and on days 2 and 5 of experiment 2, rats were housed individually in metabolic cages and 24 h urines collected.

**Tissue Preparation.** Rats were killed, and livers were immediately perfused and snap-frozen in liquid nitrogen. The colon was flushed with ice-cold isotonic saline and the first 5 cm cut open to allow the colonic mucosal cell layer to be collected by scraping. Samples were snap-frozen in liquid nitrogen and stored at −80 °C. Samples were later thawed, and microsomes and cytosol were prepared according to methods described previously, snap-frozen in liquid nitrogen, and stored at −80 °C until analysis (*18*).

**Sulforaphane Mercapturic Acid (SF-M) Analysis.** SF-M (SF *N*-acetylcysteine conjugate) was quantified by HPLC-UV. Urine samples were diluted to 20% in deionized water, and 50 μL was injected onto a Hypersil C18 reverse-phase ODS column (250 × 4.60, 10 μm, Phenomenex, Torrance, CA). A gradient solvent system, with a flow rate of 1 mL/min, consisted of a starting solvent system of 5% aqueous acetonitrile that was linearly increased to 20% over 3 min, maintained at 20% for 4 min, then raised to 100% over 2 min, and maintained at 100% for 13 min. All solvents contained 1% (v/v) of glacial acetic acid. An SF-M standard, synthesized by a modification of the method for synthesis of AITC mercapturic acid (*30*), was used both to determine the elution time and to construct a standard curve for the quantification of SF-M (**Figure 2**). The SF-M standard eluted with a peak at 10.8 min, and the structure of the urinary peak eluting at 10.8 min was confirmed as SF-M by MS-EI (Mass Spectrometry Laboratory, School of Chemical Science, University of Illinois, Urbana, IL; Hwang and Jeffery, manuscript submitted).

**Measurements of Enzyme Activities.** QR activity was measured using 2,6-dichlorophenolindophenol (DPIP) as the substrate, following the method of Ernster (*31*) as modified by Benson and co-workers (*32*). Ethoxyresorufin *O*-deethylase (EROD) activity, using ethoxyresorufin as the substrate, was estimated according to the fluorometric method of Pohl and Fouts (*33*) and taken as a measure of cytochrome P450 1A activity. Formation of resorufin was measured using an excitation wavelength of 550 nm and an emission wavelength of 585 nm and intergraded using a resorufin standard curve. Protein concentrations were measured by using the Bio-Rad assay (Bio-Rad, Hercules, CA) based on the Bradford method (*34*), using bovine serum albumin as standard.

**Statistical Analysis.** Treatment effects were determined using analysis of variance (one-way ANOVA). When a significant effect ($p \leq 0.05$) was found, Tukey's studentized range test was used to determine differences between means.

## RESULTS

**Experiment 1.** Rats fed 20% broccoli diets (HP or GS) or the purified SF diet all had significantly increased QR activity in the colon compared to rats fed the control diet (**Table 2**). Rats fed broccoli containing intact glucosinolates (broccoli-GS) had a significantly higher colonic QR activity compared to rats fed prehydrolyzed broccoli (broccoli-HP, **Table 2**), 4.6- versus 3.3-fold over control, respectively. There was no significant difference between colonic QR activities in rats fed the purified SF diet (4.6-fold) and rats fed the broccoli-GS diet (4.5-fold) even though the glucoraphanin level of the broccoli-GS diet

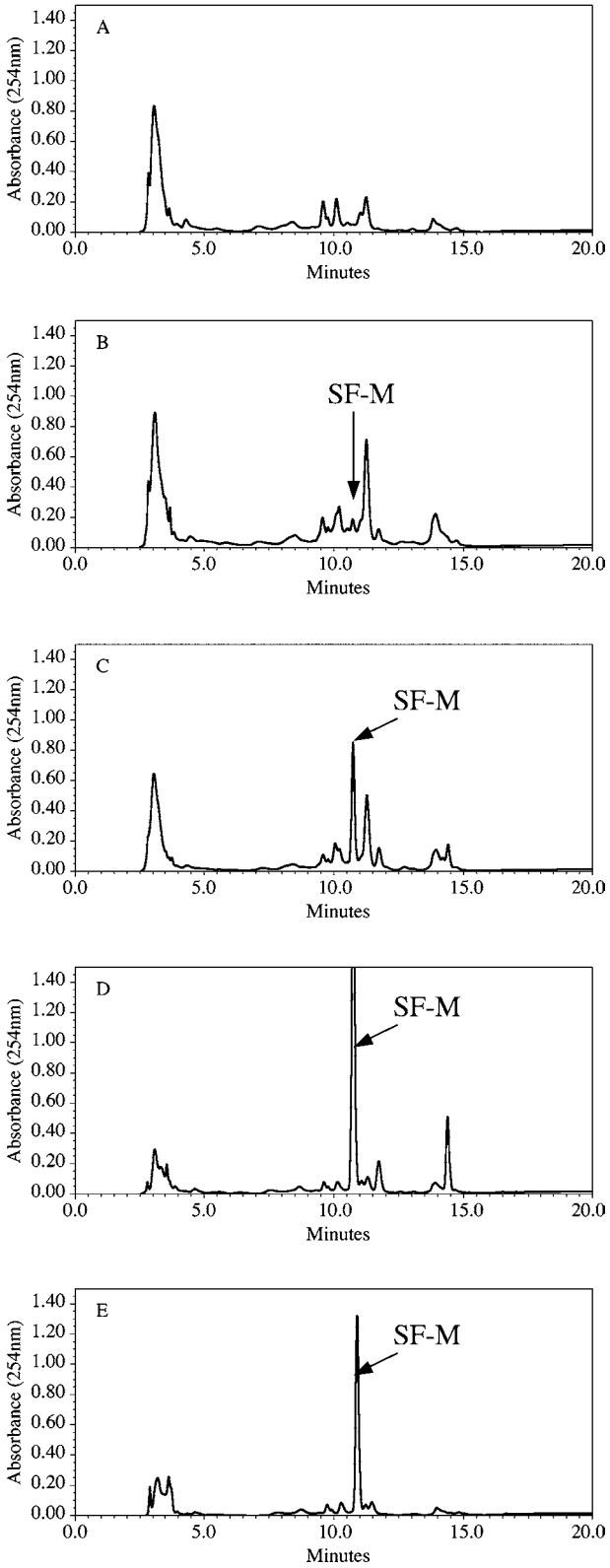


**Figure 2.** Representative HPLC chromatograms of urine from rats in experiment 1 fed a control modified AIN-76 B-40 diet (A), 20% prehydrolyzed broccoli (broccoli-HP; B), 20% unhydrolyzed broccoli (broccoli-GS; C), and purified SF (5.0 mmol/kg of diet; D). (E) Synthetic sulforaphane mercapturate (SF-M) in 20% urine (150 $\mu$g/mL). Urine was separated on a Hypersil C18 reverse-phase ODS column, and SF-M was detected by UV absorbance at 254 nm (see Materials and Methods).

(2.2 mmol/kg of diet) was <50% of the SF level in the purified SF diet (5 mmol/kg of diet). This same pattern was observed for hepatic QR activity (**Table 2**) except that the magnitude of QR induction was less and the effect of the broccoli-HP diet on hepatic QR activity was not significantly different from the control. A significantly increased hepatic EROD activity (**Table 2**) was found in rats fed both the broccoli-HP diet and the broccoli-GS diet, 1.3- and 1.4-fold over control, respectively, whereas there was no EROD induction associated with the purified SF diet.

**Table 2.** Experiment 1: Effect of Diets Containing 20% Prehydrolyzed Broccoli (Broccoli-HP), 20% Unhydrolyzed Broccoli with Intact Glucosinolates (Broccoli-GS), or Purified Sulforaphane (5.0 mmol of SF/kg of Diet) Compared to a Control Modified AIN-76 B-40 Diet (C) on Hepatic and Colonic Mucosal Quinone Reductase (QR) and on Hepatic Ethoxyresorufin *O*-Deethylase (EROD) Activities of Rats Fed These Diets for 5 Days[a]

| diet | colonic QR activity (nmol of DPIP/min/mg of protein) | hepatic QR activity (nmol of DPIP/min/mg of protein) | hepatic EROD activity (pmol of resorufin/min/mg of protein) |
|---|---|---|---|
| C | 122.1 ± 17.4a | 68.3 ± 6.1a | 116.9 ± 3.1a |
| broccoli-HP | 396.6 ± 29.6b | 77.3 ± 7.4ab | 156.6 ± 10.9b |
| broccoli-GS | 543.7 ± 33.9c | 94.4 ± 8.7b | 159.6 ± 8.5b |
| SF | 559.0 ± 43.2c | 97.3 ± 6.5b | 106.4 ± 3.4a |

[a] Values shown are means ± SE, $n = 5$. Different letters within a column indicate values that differ significantly ($p \leq 0.05$).

There was no significant difference between treatment groups in average food intake (9.4−10.0 g/rat/day) during the 5-day test period of experiment 1 (**Table 3**). However, rats fed the broccoli-HP diet had a significantly greater weight gain, 18.2 g (a 21% gain over starting weight) compared to rats fed the control diet (12.9 g, a 16% gain over starting weight) during the 5-day test period. There was a significant increase in liver weight as a percent of BW in all three treatment groups (6.4−6.7%) compared to the control (5.3%, **Table 3**).

During the last 24 h of experiment 1, urine samples were collected and analyzed for SF-M content. A peak eluting at 10.8 min, and corresponding to synthetic SF-M, was detected in urine from all rats fed broccoli or purified SF diets (**Figure 2B−D**). Peaks eluting between 11 and 12 min were seen in control and experimental urines, although they were larger in the urine of rats fed broccoli. These peaks, which are not specific to broccoli ingestion, have not been identified. Rats fed broccoli-GS had significantly more SF-M in the urine than rats fed broccoli-HP, 5.8 vs 1.1 $\mu$mol of SF-M/rat/24 h, respectively (**Figure 3**). The SF-M detected in the urine from rats fed purified SF was 20.2 $\mu$mol/rat/24 h, substantially more than that from rats fed the broccoli-HP. However, the percentage of dietary SF excreted as SF-M was greater in rats fed broccoli-HP (67%) than in rats fed purified SF (40%). The fraction of SF-M recovered in urine from rats fed broccoli-GS cannot be determined because the fraction of glucoraphanin converted to SF is not known; however, the fraction of glucoraphanin appearing as SF-M in the 24 h urine was 26%. No SF-M was detected in the urine of rats fed the control diet (**Figure 2A**).

**Experiment 2.** Rats fed broccoli-GS exhibited 1.8-fold greater colonic QR activity than rats fed a control diet (**Table 4**). When a low dose of SF (0.16 mmol of SF/kg of diet) was added to the broccoli diet, there was no greater increase in QR activity in the colon beyond that caused by the broccoli diet alone, and a low dose of SF (0.32 mmol of SF/kg of diet) alone caused no increase in QR activity (**Table 4**). A higher dose of SF (5.16 mmol of SF/kg of diet) induced QR activity in the

**Table 3.** Starting Weight, Average Daily Food Intake, Amounts of Dietary Glucoraphanin (GP) or Sulforaphane (SF), Daily Intake of GP and SF, Weight Gain during the 5 Day Test Period, and Liver Weight as a Percent of Body Weight in F344 Rats from Experiments 1 and 2[a]

| diet | starting wt (g) | food intake (g/day) | dietary GP and (SF) (mmol/kg) | intake of GP and (SF) ($\mu$mol/rat/day) | wt gain (g) | liver wt as a % of body wt |
|---|---|---|---|---|---|---|
| | | | Experiment 1 | | | |
| C | 92.9 ± 2.0a | 10.0 ± 0.2a | 0 (0) | 0 (0) | 12.9 ± 1.0a | 5.3 ± 0.3a |
| broccoli-HP | 91.8 ± 2.2a | 9.4 ± 0.4a | 0 (0.16) | 0 (1.6) | 18.2 ± 1.2b | 6.4 ± 0.2b |
| broccoli-GS | 90.9 ± 2.0a | 9.8 ± 0.2a | 2.2 (0) | 22 (0) | 15.7 ± 2.1ab | 6.7 ± 0.3b |
| SF | 89.2 ± 1.7a | 9.4 ± 0.4a | 0 (5) | 0 (50) | 11.6 ± 1.2a | 6.6 ± 0.2b |
| | | | Experiment 2 | | | |
| C | 97.8 ± 2.1a | 10.0 ± 0.1a | 0 (0) | 0 (0) | 10.4 ± 0.6a | 5.4 ± 0.2a |
| broccoli-GS | 94.8 ± 2.1a | 9.3 ± 0.3ab | 2.2 (0) | 22 (0) | 15.5 ± 1.1c | 5.8 ± 0.4a |
| broccoli-GS + 0.16 SF | 96.2 ± 1.4a | 9.0 ± 0.3ab | 2.2 (0.16) | 22 (1.6) | 15.6 ± 0.4c | 5.9 ± 0.2a |
| 0.32 SF | 94.7 ± 1.2a | 9.8 ± 0.1a | 0 (0.32) | 0 (3.2) | 11.2 ± 1.2ab | 5.6 ± 0.4a |
| broccoli-GS + 5.0 SF | 95.9 ± 2.0a | 8.3 ± 0.9b | 2.2 (5) | 22 (50) | 13.3 ± 0.4bc | 6.4 ± 0.4b |
| 5.16 SF | 95.5 ± 2.1a | 9.9 ± 0.1a | 0 (5.16) | 0 (51.6) | 11.2 ± 1.3ab | 5.6 ± 0.3a |

[a] C, control, modified AIN-76 B-40; broccoli-HP, 20% pre-hydrolyzed broccoli; broccoli-GS, 20% unhydrolyzed broccoli with intact glucosinolates; SF, purified sulforaphane, 5 mmol of SF/kg of diet. Values shown are means ± SE, $n = 5$ (experiment 1), $n = 6$ (experiment 2). Different letters within a column and within one experiment indicate values that differ significantly ($p \leq 0.05$).

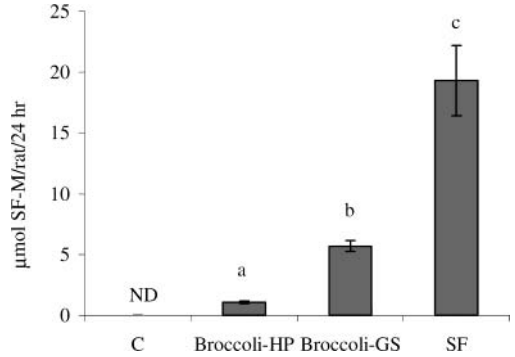


**Figure 3.** Experiment 1, day 5: amount of sulforaphane mercapturate in urine of F344 rats fed one of the following diets: control (modified AIN 76 B40), 20% prehydrolyzed broccoli (broccoli-HP), 20% unhydrolyzed broccoli (broccoli-GS), or purified sulforaphane (SF, 5.0 mmol of SF/kg of diet). Data shown are means ± SE, $n = 5$. Treatment effects were determined using one-way ANOVA and Tukey's studentized range test. Different letters indicate values that differ significantly ($p \leq 0.05$). ND = below the level of detection.

**Table 4.** Experiment 2: Colonic and Hepatic Quinone Reductase (QR) Activity in F344 Rats Fed Control, Modified AIN-76 B-40 (C), 20% Unhydrolyzed Broccoli with Intact Glucosinolates (Broccoli-GS), 20% Broccoli-GS with 0.16 mmol of Purified Sulforaphane (SF)/kg of Diet (Broccoli-GS + 0.16 SF), 0.32 mmol of Purified Sulforaphane/kg of Diet (0.32 SF), 20% Broccoli-GS with 5.0 mmol of Purified Sulforaphane/kg of Diet (Broccoli-GS + 5.0 SF), or 5.16 mmol of Purified Sulforaphane/kg of Diet (5.16 SF) in Diet for 5 Days[a]

| diet | colonic QR activity (nmol of DPIP/ min/mg of protein) | hepatic QR activity (nmol of DPIP/ min/mg of protein) |
|---|---|---|
| C | 96.0 ± 8.5b | 55.5 ± 4.4a |
| broccoli-GS | 175.8 ± 12.9c | 83.8 ± 3.6bc |
| broccoli-GS + 0.16 SF | 183.0 ± 16.9c | 62.1 ± 1.5a |
| 0.32 SF | 74.1 ± 6.6a | 57.6 ± 3.5a |
| broccoli-GS + 5.0 SF | 281.4 ± 21.4d | 99.3 ± 4.9c |
| 5.16 SF | 205.0 ± 23.2c | 78.8 ± 4.3b |

[a] Values shown are means ± SE, $n = 6$. Different letters within a column indicate values that differ significantly ($p \leq 0.05$).

colon to a similar extent as the broccoli-GS diet, 2.1- versus 1.8-fold, respectively. In rats fed broccoli-GS with a high amount of SF added (5.0 mmol of SF/kg of diet) the QR activity in the colon rose to 2.9-fold above control levels, significantly higher than either broccoli alone or the high dose of SF alone. A similar pattern in the induction of QR was seen in the liver except at a lower magnitude, and the addition of the low amount of SF (0.16 mmol/kg of diet) to the broccoli resulted in decreased hepatic QR induction relative to the broccoli diet alone (**Table 4**).

Rats that received the broccoli diet with the added high SF dose had significantly lower food intake than rats receiving the control diet, although intake was not significantly different among groups receiving the various broccoli diets. Nonetheless, rats receiving the broccoli diets gained significantly more weight (13.3−15.6 g, a 13−16% gain over starting weight) than rats receiving the control diet (10.4 g, an 11% gain over starting weight) or SF diets (11.2 g, a 12% gain over starting weight). The liver weights as a percent of BW were similar in all animal groups except those rats that received the broccoli + high SF diet, which had significantly larger livers, 6.4% compared to 5.4% for control rats.

As in the first experiment, urine from all groups receiving diets containing either broccoli or SF, even the lowest SF content (0.32 mmol/kg of diet), contained measurable amounts of SF-M (**Figure 4**). Twenty-four hour excretion is reported separately for days 2 and 5, although results were very similar in all but the group fed 0.32 mmol of SF/kg of diet, in which SF-M increased significantly from 1.0 to 1.9 $\mu$mol of SF-M/rat/24 h. On day 2, the percentage of dietary SF that appeared in the urine as SF-M was 35% for the low-SF diet and 39% for the high-SF diet. On day 5, the percentage of dietary SF excreted into the urine, as SF-M, was 59% for the low dose of SF and 37% for the high dose of SF. No SF-M was detected in urine from rats given the control diet. Rats fed the low dose of SF plus broccoli had decreased amounts of SF-M in the urine compared to rats fed broccoli alone. When the broccoli diet was supplemented with the high dose of SF, no great increase in SF-M was detected in the urine compared to pure SF alone.

**Table 5** illustrates the relationship between SF-M excreted into urine and the increase of QR activity in the liver and colon from both experiments. In both experiments 1 and 2 there was significant linear correlation between SF-M and QR activity in the liver ($p = 0.032$ and 0.001, respectively) and colon ($p = 0.032$ and $<0.0001$, respectively).

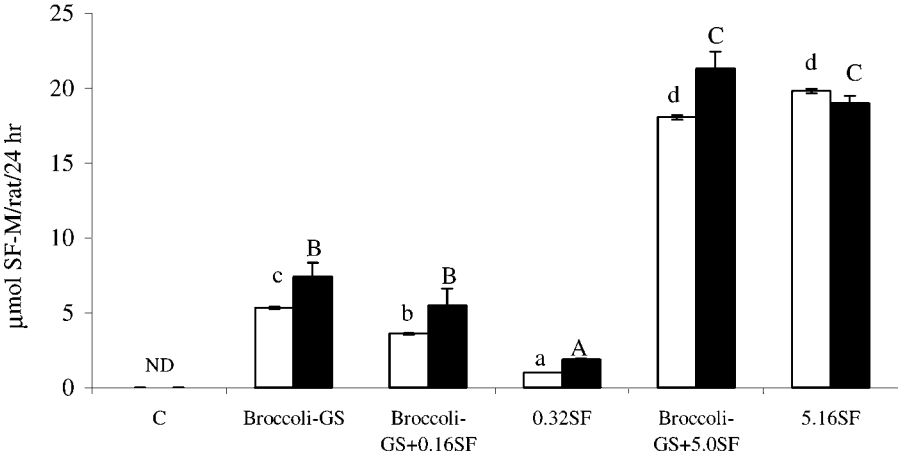


**Figure 4.** Experiment 2: amount of sulforaphane mercapturate in urine on days 2 (white bars) and 5 (black bars) from F344 rats fed one of the following diets: control modified AIN-76 B-40 (C), 20% unhydrolyzed broccoli (broccoli-GS), 20% broccoli + 0.16 mmol of SF/kg of diet (broccoli-GS + 0.16 SF); 0.32 mmol of SF/kg of diet (0.32 SF), 20% broccoli + 5.0 mmol of SF/kg of diet (broccoli-GS + 5.0 SF), or 5.16 mmol of SF/kg of diet (5.16 SF). Values shown are means ± SE, $n = 3$. Treatment effects were determined using one-way ANOVA and Tukey's studentized range test. Different letters (lower case for day 2 and capitalized for day 5) indicate values that differ significantly ($p \leq 0.05$). ND = below the level of detection.

**Table 5.** Experiments 1 and 2: Pearson Correlation Coefficient Quinone Reductase (QR) Activity in Liver or Colon with 24 h Urinary Excretion of Sulforaphane Mercapturic Acid (SF-M) during Day 5

| | SF-M |
|---|---|
| experiment 1 | |
| QR liver | 0.4812 (0.032)[a] |
| QR colon | 0.5552 (0.011) |
| experiment 2 | |
| QR liver | 0.7266 (0.0006) |
| QR colon | 0.8134 (<0.0001) |

[a] Values in parentheses are *p* values.

## DISCUSSION

Here we show, for the first time, that dietary broccoli containing intact glucosinolates (broccoli-GS) increased rat QR activities in the liver and colon to a substantially greater extent than broccoli containing SF preformed as a product of myrosinase-dependent glucosinolate hydrolysis (broccoli-HP, **Table 2**). Furthermore, purified SF is less effective than broccoli, on a mole for mole basis. These data raise important issues about the formation and absorption of SF that need to be taken into consideration in planning and implementing the processing and preparation of broccoli and dietary supplements from broccoli for optimal health benefits.

The cause for the poor QR-inducing activity of the prehydrolyzed broccoli compared to the unhydrolyzed broccoli appears to be that broccoli-HP provides less SF than broccoli-GS, because urinary SF-M was 5-fold greater in rats fed broccoli-GS than in those fed broccoli-HP. This difference was not due to SF loss from broccoli-HP before ingestion: we analyzed broccoli powders after hydrolysis, after freeze-drying the hydrolyzed product, after rehydrolysis, and following storage in feeders for 24 h at room temperature and found no difference in SF content among broccoli samples.

The amount of SF-M excreted by rats fed broccoli-GS (5.5 μmol/rat/24 h) was several times greater than the intake of SF in the rats receiving broccoli-HP (1.6 μmol/rat/24 h), so this difference cannot be explained by a greater absorption of SF from broccoli-GS. Furthermore, if absorption was even a partial cause for the difference between the two diets, this difference would have been absent or severely diminished in the colon, where colonic mucosal cells are exposed to bioactive components directly as well as systemically. We found that induction due to broccoli-GS was considerably greater than induction due to broccoli-HP, in both liver and colon. These data are consistent with a greater yield of SF during hydrolysis of broccoli-GS compared to broccoli-HP, not with a greater absorption. We also found that for both preparations, QR induction was significantly greater in the colon than in the liver. These data support the idea that the beneficial effect from dietary broccoli may be greater in the colon than in organs reached only systemically.

The broccoli-GS powder had not been heated to destroy endogenous plant myrosinase activity and, therefore, it is possible that hydrolysis of glucosinolates was catalyzed by the plant enzyme present in the powder and that SF production was favored over other hydrolysis products in broccoli-GS by the environment in the gut. Environmental factors such as temperature and pH are known to alter the ratio of glucosinolate hydrolysis products in vitro (*6*). However, an acid environment is reported to favor nitrile formation over SF formation, making the drop in pH within the stomach environment an implausible explanation for the increased SF yield.

In addition to hydrolysis facilitated by myrosinase, glucosinolates may be hydrolyzed by the colonic microflora. Gnobiotic rats, lacking microflora because they have been treated with oral antibiotics, had no increase in hepatic detoxification enzymes following ingestion of a high-glucosinolate rapeseed diet (*35*). When humans were fed cooked broccoli, only those who had not received oral antibiotics were found to have isothiocyanates in their urine (*36*). There are no published studies to date reporting products formed during microbial-dependent hydrolysis of glucoraphanin.

The present data suggest that the yield of SF is far greater during in vivo hydrolysis in the presence of both broccoli myrosinase and gut bacteria than during broccoli hydrolysis in situ, which is dependent solely upon the endogenous broccoli myrosinase. There is one study comparing the excretion of SF-M in humans following ingestion of steamed and raw broccoli (*27*). In that study, the authors conclude that myrosinase, rather than bacterial hydrolysis, plays a critical role in the conversion of ingested glucosinolates to isothiocyanates, because SF-M excretion was 3−4-fold greater following ingestion of fresh broccoli compared to ingestion of steamed broccoli. Their calculated yield of SF-M from dietary glucoraphanin was substantially

smaller than ours, being 12% (fresh broccoli), whereas ours was 26% (broccoli-GS), which may be due to species differences between rats and humans. They used an exogenous myrosinase from white mustard (Sigma) to analyze the broccoli for SF and used this to calculate yields. Myrosinase extracted from white mustard is not associated with nitrile formation (*37*, *38*). Therefore, their calculated starting amount of SF, although accurately measuring dietary glucoraphanin, greatly overestimates the yield of SF produced during hydrolysis with endogenous broccoli myrosinase.

Rats fed broccoli-HP excreted 67% of their dietary SF as SF-M, whereas rats fed purified SF excreted only 40% as SF-M. Thus, bioavailability of SF was better from the broccoli matrix than from pure SF. However, formation of SF during in situ hydrolysis was extremely poor (6%). Therefore, whereas purified SF is less bioavailable that SF within broccoli, in situ glucoraphanin hydrolysis provides a sufficiently poor yield of SF as to negate this advantage when availabilities from purified SF or hydrolyzed whole broccoli are compared. It remains to be determined how one might optimize hydrolysis to SF, as occurs when exogenous myrosinase is added (*36*), and harness the improved bioavailability of SF within broccoli that we saw with the broccoli-HP powder.

This is the first report of urinary SF-M excretion in rats receiving purified SF in the diet. When rats were injected ip with purified SF (50 $\mu$mol/rat), 60% of the dose was excreted as SF-M (*26*). In this study, only ~40% of dietary SF was found to be excreted as SF-M. The importance of this lower excretion as SF-M is that SF-M is indicative of the bioactivity of SF (**Table 5**). In rats receiving a 16-fold lower dose, excretion of SF-M was proportionately 16-fold lower (**Figure 3**), suggesting that lower recovery of the larger dose was not due to saturation of the system. This finding may help our understanding of the unexpectedly low response in QR activity found by other researchers using purified SF administered orally (*14*, *15*).

Under the experimental conditions used, the broccoli matrix did not facilitate the uptake of exogenous SF when broccoli and purified SF were given together in the diet. Addition of the purified exogenous SF to a broccoli diet may not have correctly modeled interactions, which might be enhanced by compartmentalization within the broccoli tissue. A small amount of purified SF (0.32 mmol/kg of diet) did not induce QR activity even though SF-M was detected in the urine at an amount similar to the amount detected following ingestion of broccoli-HP, which was associated with bioactivity. This suggests that the amount of purified SF absorbed was similar to that associated with bioactivity when present in broccoli, but it appeared to be less active than broccoli, supporting a role for additional bioactive components within broccoli.

Whereas SF is the major bioactive component of broccoli (*13*, *14*), there are multiple other bioactive components present in broccoli that might account for the difference in potency between purified SF and broccoli. Broccoli-HP and GS, but not purified SF, induced hepatic CYP1A activity as measured by EROD activity, supporting the assumption that there are other bioactive compounds, such as indole-3-carbinol, present in broccoli in addition to the monofunctional inducer SF. In addition to other glucosinolate hydrolysis products, there are flavonoids such as quercetin and other bioactive compounds, including *S*-methylcysteine sulfoxide (*39*), many of which have been proposed to play a role in the ability of broccoli to induce QR activity (*40*). However, although this might be a minor factor, it is unlikely to be the major factor describing the differences seen, because we found that in all rats, regardless of whether they were fed pure SF, broccoli-GS, or broccoli-HP, the extent of QR inducibility correlated with SF-M, suggesting that, to a great extent, the amount of SF absorbed accounts for the QR activity. Induction of QR activity in the colon and liver correlated significantly with the amount of SF-M detected in the urine in both experiments (**Table 5**). These data suggest that SF-M is proportional to circulating SF and/or other components causing QR induction when rats are fed broccoli, making SF-M perhaps an excellent biomarker for QR induction in the colon and liver.

In summary, feeding broccoli containing intact glucosinolates provided greater QR inducibility than broccoli hydrolyzed prior to feeding. The cause for this appears to be related to improved hydrolysis of glucoraphanin to SF. Although neither endogenous nor bacterial hydrolysis of glucoraphanin gives as good a yield of SF from glucoraphanin as the exogenous white mustard myrosinase, the broccoli matrix greatly enhances SF uptake compared to absorption of purified SF. Urinary SF-M has the potential to be a good biomarker for the effects of dietary broccoli on the induction of QR activity in liver and colon and by extrapolation, a measure of relative cancer preventative effects of broccoli diets.

## ABBREVIATIONS USED

AITC, allyl isothiocyanate; DW, dry weight; DPIP, 2,6-dichlorophenolindophenol; EROD, ethoxyresorufin *O*-deethylase; GC, gas chromatography; GS, glucosinolates; GST, glutathione *S*-transferases; HP, hydrolysis products; HPLC, high-performance liquid chromatography; SF, sulforaphane; SF-M, sulforaphane mercapturate; SE, standard error; QR, quinone reductase

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