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Betalains, Phase II Enzyme-Inducing Components From Red Beetroot (*Beta vulgaris* L.) Extracts

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Abstract: *Crude aqueous and ethanolic extracts of root tissue of red (Rd) and high-pigment (HP) beet (*Beta vulgaris *L.) strains exhibited antioxidant and phase II enzyme-inducing activities, and these extracts were fractionated using Sephadex LH-20 chromatography. These bioactivities tended to become co-enriched in early and late eluting fractions, comprising 5–25% of the material recovered from the column. Liquid chromatography-mass spectrometry (MS) was used to resolve and identify multiple betalain components in the most potent quinone reductase (QR)-inducing fractions. Active fractions were found to contain vulgaxanthins I and II, and (iso)betanin, but other components remained unidentified. Two of the isolated active fractions were incorporated into rodent diets at 10–150 ppm over a 2-mo period to assess bioavailability and in vivo efficacy for phase II enzyme induction in various organs. No statistically significant effect of diet was obtained, and wide ranges of tissue enzyme levels among individual animals were observed. This lack of effect and diversity in response to diet may be related to the wide range in absorptive capacity of and/or insufficient level or enrichment of the active agents or to difficulties in assessing such activity in vivo. Subsequent to the animal studies, betanin was isolated in pure form, identified by MS analysis, and confirmed to be QR inducers in the bioassay.*

Introduction

Red (Rd) beetroots are grown mainly in Europe, parts of Asia, the United States, and the Mediterranean region (1). They are not considered to be a popular vegetable in North America, and per capita consumption of beet makes it among the least consumed vegetables (2). However, Rd beetroots are an excellent source of red and yellow pigments, and this trait has fostered research and industrial interest in the context of natural colorants (1,3,4).

Beet pigments, collectively known as betalains, have been examined as natural colorants in food products such as processed meat, ice cream, baked goods, candies, and yogurt (4–7). There are two distinct classes of betalains, the red/purple betacyanins and the yellow betaxanthins (4,8). Besides imparting attractive color to food products, betalains have been shown to confer free-radical scavenging and allied antioxidant activities (2,9–12). Crude betalain preparations may also contain various phenolic acids and their esters that could contribute to the antioxidant potential and other bioactive effects observed for such preparations (3,13–17).

It is widely believed that diets rich in fruits and vegetables are associated with decreased cancer risk (18). One mechanism by which cancer chemoprevention may be conferred is through the up-regulation of phase II detoxifying enzymes by dietary constituents. Using a predictive bioassay for phase II enzyme induction, based on cultured murine hepatoma cells (19), we previously demonstrated that beetroot crude extracts contained multiple antioxidant and phase II enzyme-inducing activities (20). However, the aqueous and 95% ethanol (5% aqueous) extracts of only the red- but not orange- or white-colored beetroot phenotypes exhibited phase II enzyme-inducing activities, and the red phenotypes were also richer in multiple antioxidant functions.

Antioxidants have also been implicated in cancer chemoprevention due mainly to their direct involvement in eliminating carcinogens, such as free radicals and genotoxic electrophiles (21). Many antioxidants have the ability to induce phase II detoxifying enzymes, which include quinone reductases (QRs) and glutathione *S*-transferases (GSTs) (22,23). Although antioxidant functions and phase II enzyme-inducing activities were enriched in beetroot extracts containing betacyanins (20), these bioactivities may be derived from a host of other constituents that may be present in these crude extracts, such as phenolic acids and their esters, flavonoids, aromatic peptides, and other naturally occurring antioxidants.

As an extension of our previous study (20), we tested in vivo efficacy of phase II enzyme induction conferred by partially purified beetroot preparations in the diets of rodents and sought to isolate and identify the constituents in crude extracts of Rd beetroots exhibiting this bioactivity in vitro.

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Materials and Methods

Materials

Beetroots used in this study were among those used in a previous study (20). These beets were breeding material derived from crosses of inbred lines released by the University of Wisconsin Table Beet Breeding Program (24) and provided by Irwin Goldman of the Department of Horticulture, University of Wisconsin–Madison. Chemicals and biochemical and biological reagents used for antioxidant and QR assays and culture media were sourced as reported (20). Sephadex LH-20 was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO) and organic solvents were obtained from Fisher Scientific (Chicago, IL).

Preparation of Crude Extracts and Fractions

Crude aqueous and 95% ethanol (5% aqueous) extracts of Rd and high-pigment (HP) red/purple strains of beetroots were prepared as described previously (20). Briefly, powdered, dried beetroot tissue was extracted (1/10, wt/vol) in boiling water for 2 min or for 25 min in 60°C ethanol (1/6, wt/vol). Removal of extraction medium by lyophilization and/or rotary evaporation was used to obtain the dry matter representing the crude extracts.

In Vitro Assays for Antioxidant and Phase II Enzyme-Inducing Activities

Inhibition of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical cation ($ABTS^{\bullet+}$) generation: This assay was conducted in sodium phosphate-buffered saline (PBS, pH 7.4) and was based on the ability of test isolates to inhibit the metmyoglobin/H_2O_2-mediated oxidation of ABTS to $ABTS^{\bullet+}$ followed by an increase in absorbance at 734 nm (25) over a 10-min period as previously described (20). Degree of inhibition was determined as a reduction in area under the curve (calculated using SigmaPlot® 2000 software for Windows, version 6.00, Jandel Scientific, San Rafael, CA) relative to a noninhibited control sample.

Measurement of reducing power: This assay was based on the ability of test isolates to reduce preformed $ABTS^{\bullet+}$ in PBS (pH 7.4) as previously described (20). Relative reducing power (0–100) was determined as the degree to which the initial absorbance at 734 nm of the solution (~0.83) was quenched by the end of a 10-min incubation period.

Inhibition of peroxyl radical-mediated β-carotene bleaching: This assay was based on the ability of test isolates to inhibit decolorization of β-carotene (absorbance at 452 nm) at 50°C mediated by an azo-polymerization catalyst in the presence of linoleic acid as previously described (20). Degree of inhibition was determined as the extent to which the initial absorbance at 452 nm was maintained after a 5-min incubation period relative to a noninhibited control.

Bioassay of phase II enzyme-inducing potency: The "Prochaska" bioassay is based on the induction of QR-specific activity in murine hepatoma (Hepa 1c1c7) cells cultured in 96-well microtiter plates (19, reviewed in Ref. 26). Test isolates were added to wells containing adherent cells (10^5 cells/well) in a serially diluted fashion in duplicate plates followed by an incubation ("induction") period of 48 h. Cells were then lysed, and QR activity was measured in one plate and protein levels were measured in the other plate by crystal violet staining. QR-specific activity was not estimated where decreases in cell protein (proportional to losses in cell viability) exceeded 50% relative to noninduced control samples. QR induction was then calculated as the ratio of QR-specific activity in the treated (induced) sample relative to the control sample.

Sephadex LH-20 Column Chromatography

Lyophilized material (500 mg dry matter) originating from crude aqueous and ethanolic extracts was dissolved in 3 ml of 50% (vol/vol) aqueous methanol and 3 ml of 95% (vol/vol) methanol (5% water), respectively, to render all extracted material visibly soluble. These reconstituted extracts were then applied to a column (2.5 cm × 75 cm) packed with Sephadex LH-20 (particle size 25–100 μm) and eluted with either 50% or 95% methanol. Eluting fractions (4 ml) were obtained with a fraction collector (Foxy Jr., ISCO, Inc., Lincoln, NE), and absorbance at 280 nm was used to construct elution profiles. The collected material was pooled into major fractions I–IV for aqueous extracts and I–V for ethanolic extracts. Methanol was evaporated under vacuum at 40°C, and the resulting aqueous preparations were frozen and lyophilized for 48 h at 13.3 Pa. Lyophilized fractions were stored in amber glass bottles at 4°C until used.

High-Performance Liquid Chromatography-Electrospray Ionization-Mass Spectrometry

Active QR-inducing fractions were subjected to liquid chromatography-mass spectrometry (LC-MS) analysis. Positive electrospray ionization (ESI)-MS was recorded using a PE SCIEX AP1 365 LC-MS system (Applied Biosystems, Foster City, CA) equipped with a C_{18} column (100 mm × 2 mm i.d., 4μm; Phenomenex, Columbus, OH). In the scanning mode, mass data were collected between mass-to-charge ratios (*m/z*) 100 and 1,000 at 0.1-amu steps. A linear gradient elution program from 10% (vol/vol) to 50% acetonitrile in 0.2% (vol/vol) aqueous acetic acid was used over the first 10 min followed by isocratic elution at a flow rate of 70 μl/min (8). Injection volume was 2 μl.

Purification and Identification of Betalains in QR-Inducing Isolates

The same Sephadex LH-20 column described earlier was used to resolve pigments in an aqueous extract of HP beetroot. Elution by water afforded five pigmented and two nonpigmented fractions. Further purification of the most abundant QR-inducing betalain fraction was achieved by preparative LC (Discovery C18, 250 mm × 21.2 mm, 5 µm; Supelco, Bellefonte, PA) by isocratic elution with aqueous 5% acetonitrile with 0.5% formic acid at 7 ml/min. Elution was monitored by absorbance at 280 nm, and presumptively pure components were recovered and lyophilized prior to analysis for in vitro QR induction and ultraviolet-visible (UV-Vis) and mass spectra.

Analytical LC using the same mobile and stationary phases (250 mm × 4.6 mm, 5-µm column) was used with diode array detection (1100 Series LC system, Agilent, Wilmington, DE) to obtain UV-Vis spectra and profile the purified components as well as the betalain-containing Sephadex LH-20 fraction of origin.

Orthogonal pneumatically assisted ESI-MS was recorded in the positive ion mode using an Agilent MSD SL quadrupole mass spectrometer. Samples were directly infused at 5 µl/min in 0.1% acetic acid, and mass data were collected between *m/z* 100 and 1,000 at 0.1-amu steps. Tandem MS (MS-MS) analyses of selected and isolated *m/z* fragments were conducted to obtain daughter ion spectra.

Animal Studies

Post-weaning male Sprague-Dawley rats (age 4 wk, 80–100 g) were obtained from Harlan Teklad (Madison, WI). For 2 wk after arrival, rats were fed (ad libitum) and conditioned on a standard experimental diet (modified AIN-93G diet), where DL-α-tocopherol acetate (37.8 IU/kg) and ascorbic acid (28 mg/kg) replaced *tert*-butyl hydroquinone (TBHQ) because TBHQ is known to be an effective phase II enzyme inducer (21,23,27). After this conditioning period, rats were randomly divided into three dietary groups. One group was continued on the control diet for an additional 2 mo. A second group was placed on a diet supplemented with one betalain isolate (10 ppm aqueous HP beetroot fraction IV) for an additional 2 mo and designated diet "A" for the balance of this article. A third group was placed on a diet supplemented with another betalain isolate (150 ppm aqueous HP beetroot fraction I), designated diet "B", for an additional 2 mo. All modifications of the AIN-93G diet were compensated for by adjustments in the amount of the corn starch (<0.1% difference between diets).

Rats had free access to food and water, and body weight was measured at intervals of ~2–3 days to monitor food intake and weight gain. Rats were housed in individual cages at the animal facilities of the Department of Animal Science, University of Wisconsin–Madison, in a temperature-controlled room (22°C) with a 12–12 h dark–light cycle (07:00–19:00 light, 19:00–07:00 dark). Animal care and use protocols were approved by the University of Wisconsin–Madison Institutional Review Board. To minimize diurnal variation, rats were sacrificed starting at ~2 h after the beginning of the light cycle and within a 3-h period (09:00–12:00) by injection of sodium pentobarbital (120 mg kg^{-1}). The abdominal cavity of the animal was quickly opened, and after exsanguination a portion of the liver, proximal small intestine, colon, kidney, and lung was excised, rinsed in cold (4°C) 0.05 M Tris-HCl buffer (pH 7.5) containing 0.25 M sucrose and 1 mM ethylenediaminetetraacetic acid (EDTA), clamped and frozen in liquid N_2, and then stored at –80°C until the tissue was analyzed (within 30 days).

Tissue Analyses

Thawed tissues were homogenized in 30 volumes of 0.05 M Tris-HCl buffer (pH 7.5) containing 0.25 M sucrose and 1 mM EDTA at 4°C with a Potter-Elvehjem (Teflon) tissue homogenizer using a Servodyne overhead mixer drive (Cole-Palmer, Vernon Hills, IL) at top speed (listed as 900 rpm). Different numbers of cycles or strokes (of 4–5 s each) were used to disrupt tissues judged to achieve a similar visual degree of homogenization: liver and kidney, 10; lung, 15; intestine, 22; and colon, 25. Homogenates were centrifuged at 2,900 *g* for 20 min and then 18,100 *g* for 60 min (all at 4°C), with the final supernatant fraction taken for enzyme and protein assays. QR was assayed in microtiter plates as described previously. GST activity was measured as initial rates of change in absorbance at 340 nm (ε 9.6 mM^{-1} cm^{-1}) using 1.0 mM 1-chloro-2,4-dinitrobenzene and 1.0 mM glutathione (28) as adapted to microtiter plates (29). Protein was measured using the Coomassie blue dye-binding method (30). Enzyme assays were conducted using an optical microtiter plate scanner (Spectra Max plus, Molecular Devices, Sunnyvale, CA), where initial reaction rates were estimated and results reported as specific activities.

Statistical Analysis

For antioxidant functions, results are presented as the mean ± SD for triplicate samples. Results were analyzed by one-way analysis of variance followed by Tukey's studentized range test (31) using a value of $P < 0.05$ to establish significant differences.

Analysis of the animal feeding trials was by the general linearized model employing type III sum of squares analysis (SAS version 8.02, SAS Institute, Inc., Cary, NC).

Results and Discussion

Fractionation of Crude Solvent Extracts by Sephadex LH-20 Chromatography

Chromatography of aqueous and ethanolic crude extracts of Rd and HP beetroot powder revealed different elution pro-

Table 1. Relative Yields and Appearance of Column Chromatographic Fractions of Aqueous and Ethanolic Extracts of Beetroots

	Red Beetroot Extracts[a]		High-Pigment Beetroot Extracts[a]	
Fraction	Aqueous	Ethanolic	Aqueous	Ethanolic
I	5.63 (red)[b]	2.80 (red)	14.0 (red)	2.60 (red)
II	86.8 (orange)	90.1 (yellow)	73.0 (orange)	83.0 (yellow)
III	4.46 (brown)	2.50 (brown)	1.50 (brown)	1.40 (brown)
IV	3.11 (purple)	3.20 (purple)	11.5 (purple)	13.0 (purple)
V	—	1.40 (black)	—	trace (black)
∑ I–V[c]	84	82	93	91

a: Recoveries are expressed in terms of % (wt/wt) of material recovered from 500 mg of the corresponding crude aqueous or ethanolic extract applied to the column.
b: Color or appearance of fraction.
c: Total % recovery (wt/wt) of 500 mg loaded on the column.

files for each of the four samples prepared (data not shown). Pooling into fractions was rather arbitrary, but the attempt was made to generally resolve eluting material by monitoring absorbance at 280 nm. This approach resulted in general consistency among the isolated fractions from each sample in terms of fractional recoveries and appearances (Table 1). Recovery of material designated a priori as fraction V obtained from the ethanolic extract of HP beetroot powder was of insufficient quantity to characterize further. Total recovery of applied material ranged from 82 to 93% (dry matter basis).

Based on appearance of the fractions, betalains are likely to be enriched in fraction I as well as fractions IV/V. The more apolar betalain species (acylated or nonglycosylated) may be enriched in the latter fractions (8,32), and these fractions were especially abundant in extracts of the HP beetroot tissue. Some oxidation/polymerization of endogenous phenolics (including betalains) and flavonoids likely occurred during isolation as inferred from the characteristic coloration of fractions III and V.

Fractionation of Antioxidant Activity by Sephadex LH-20 Chromatography

Consistency was observed among isolated fractions from each extract in terms of antioxidant activity in the three assays employed. Antioxidant activity, indexed as inhibition of heme-H_2O_2–mediated oxidation, radical reducing power, and inhibition of peroxyl radical-mediated oxidation (Fig. 1), was most enriched in fraction I and then fraction IV obtained from crude aqueous extracts of Rd beetroot powder and crude ethanolic extracts of HP beetroot powder. These same antioxidant activities were most enriched in fractions I and III resolved from crude aqueous extracts of HP beetroot powder and crude ethanolic extracts of Rd beetroot powder. All other fractions obtained were of lesser antioxidant potency in these assays, and in some cases no antioxidant activity could be observed (not statistically different from noninhibited controls). The largest fraction recovered in all cases (fraction II) was either the least effective antioxidant preparation or ineffective as an antioxidant.

Beetroot crude extracts are rich in antioxidant power compared with other vegetables when assessed by a broad range of antioxidant assays (2,12,33,34). In some studies, antioxidant function has been attributed directly to betalains in terms of reducing power (9,35) and inhibition of iron-induced lipid peroxidation (10,11). The observation that antioxidant activities extracted from beetroots were enriched in fraction III (Fig. 1 and Table 1) in addition to fraction I (presumptively betalains) provides an avenue for isolating and characterizing non-betalain antioxidant components endogenous to beetroot tissue.

Fractionation of Quinone Reductase–Inducing Activity by Sephadex LH-20 Chromatography

Fraction I from aqueous extracts of Rd beetroot tissue was the only fraction capable of inducing QR in Hepa 1c1c7 cells (Fig. 2A). Fractions I and V from ethanolic extracts of Rd beetroot were capable of inducing QR activity, whereas fraction IV suppressed QR activity in the Hepa 1c1c7 cell line (Fig. 2B). Fractions II, IV, and V from the ethanolic extracts of Rd beetroot tissue caused reductions in cell densities (loss in cell viability), whereas all other fractions had no effect on cell density relative to untreated controls (data partially shown in Fig. 2A and B). Thus, only fraction I from both aqueous and ethanolic extracts of Rd beetroot tissues possessed both QR-inducing (Fig. 2A and B) and broad antioxidant (Fig. 1) activities.

For both aqueous and ethanolic extracts of HP beetroot powder, fractions I and IV were capable of inducing QR in Hepa 1c1c7 cells (Fig. 2C and D). Fraction III isolated from the ethanolic extract was also capable of QR induction, and it appeared that fraction III from the aqueous extract may have been able to double QR-specific activity if sufficient material was available to extend the dose-dependent response (fraction III from HP beetroot tissues was the least abundant isolate, Table 1). Only fraction IV from the ethanolic extracts of HP beetroot powder caused a significant reduction (>50%) in cell density relative to untreated

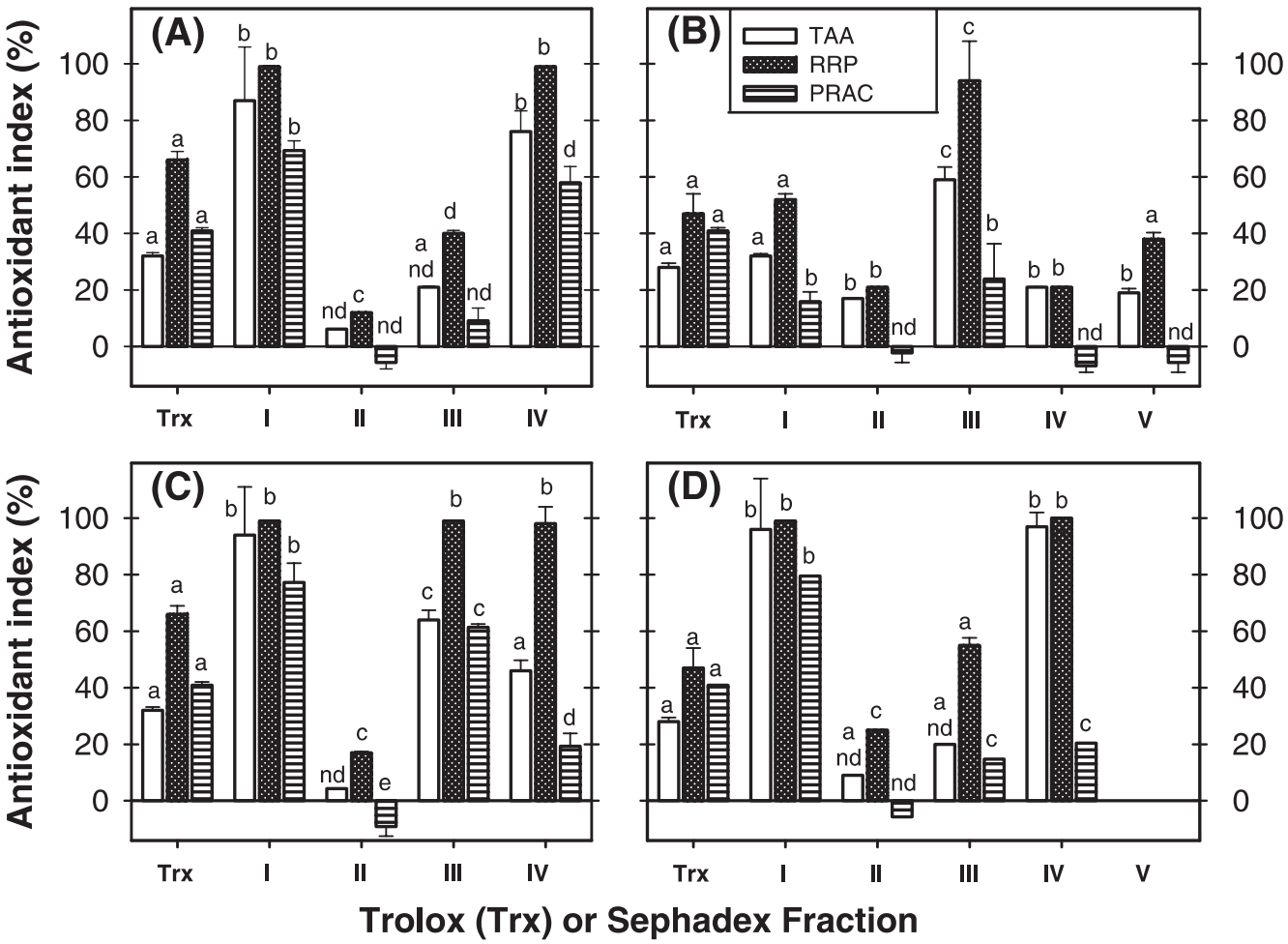

Figure 1. Antioxidant activities of crude fractions obtained from beetroot extracts. The antioxidant index is used to express relative antioxidant effectiveness in each assay where "0" represents the response of a "control" (without antioxidant) and "100" represents complete antioxidant effectiveness in the assay. Total antioxidant activity (TAA) represents inhibition of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$) generation; radical reducing power (RRP) represents the ability to reduce or bleach $ABTS^{\bullet+}$; peroxy-radical absorbing capacity (PRAC) represents the inhibition of β-carotene bleaching. Fractions are those obtained from Sephadex LH-20 chromatography from (A) crude aqueous extract of red (Rd) beetroot tissue, (B) crude ethanolic extracts of Rd beetroot tissue, (C) crude aqueous extract of high-pigment (HP) beetroot tissue, and (D) crude ethanolic extracts of HP beetroot tissue (insufficient material available to assess fraction V; see Table 1). Results are expressed as means ± SD from triplicate determinations. Means accompanied by different lower case letters within each panel for the same antioxidant assay are different ($P < 0.05$), and those designated "nd" are not different from the control.

controls, and this was responsible for the inability to extend the dose-response range for this fraction beyond 0.125 mg/ml (Fig. 2D). Thus, generally fractions I, III, and IV from both aqueous and ethanolic extracts of HP beetroot tissue possessed both QR-inducing (Fig. 2C and D) and broad antioxidant (Fig. 1) activities, with fraction IV from both crude extracts being most effective at QR induction and fraction I being most effective at antioxidant function. The link between antioxidant function and phase II enzyme induction is not surprising given the emerging view that a redox sensing element in the cell is responsible for up-regulation of phase II enzymes (23,27,36–38).

Tentative Identification of Components in Quinone Reductase–Inducing Fractions

Based on the combined observations of greatest QR-inducing potency in the Hepa 1c1c7 bioassay and relative abundance of isolated fractions, only fractions I, IV, and V from crude extracts of Rd and HP beetroot powder were subjected to attempts to further resolve and identify active agents. These fractions were red-purple-black, implicating betalains and perhaps betalain derivatives as major components or markers for the bioactive agents. The most abundant betalains in beetroot are the red betacyanin betanin

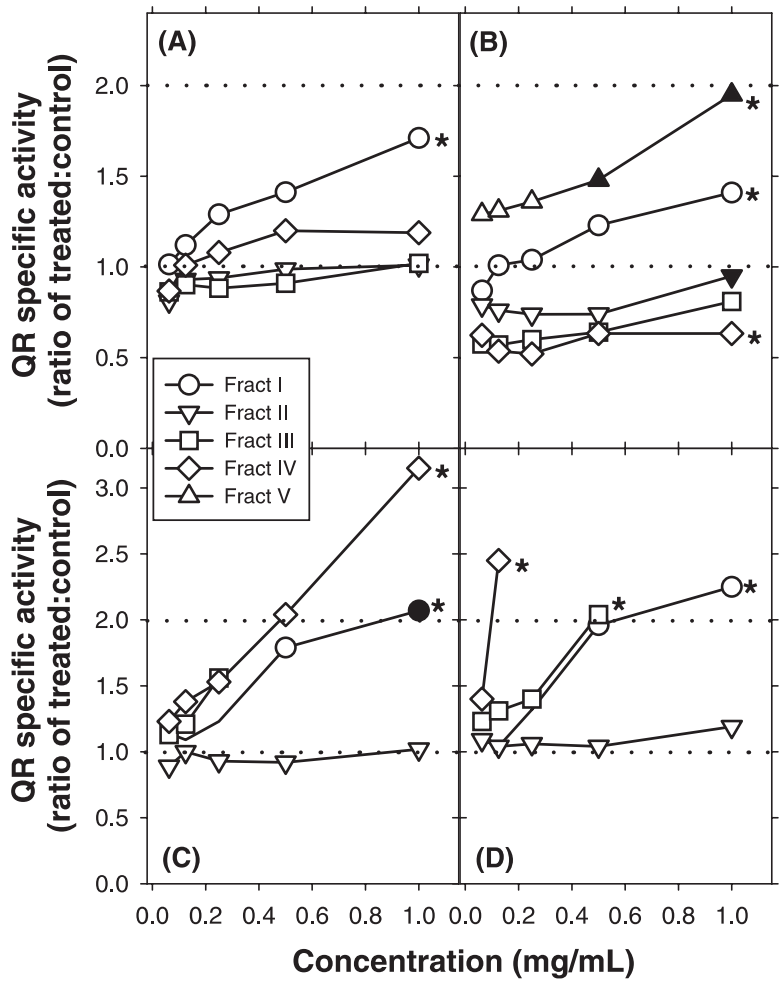

Figure 2. Quinone reductase induction in Hepa 1c1c7 cells by Sephadex LH-20 fractions obtained from crude extracts of beetroot tissue. Fractions were obtained from crude aqueous (A) and ethanolic (B) extract of red beetroot tissue and aqueous (C) and ethanolic (D) extracts from high-pigment beetroot tissue. Results are expressed as means ± SD from triplicate determinations. Dose-dependent responses for fractions marked by an asterisk within each panel were different ($P < 0.05$) from the noninduced controls. Portions of the dose-dependent responses where loss of cellular protein (viability) was 20–50% relative to controls appear as closed symbols.

(Fig. 3C) and the yellow betaxanthin vulgaxanthin I (Fig. 3A), both of which are aldimine conjugation products of betalamic acid (Fig. 3B) and an amino acid, cyclo-dihydroxyphenylalanine and glutamine, respectively (17,39). Isobetanin is also present at levels ranging from 0.6 to 41% of the levels of betanin in beetroot (40) and is a stereoisomer of betanin at C-15.

Fraction I obtained from the aqueous extract of red beetroot powder did not yield sufficiently strong signals from liquid chromatography-mass spectrometry (LC-MS) analysis to permit identification of betalain components (Table 2). This may have been caused by the relative abundance of sugars and aliphatic acids in this fraction, effectively diluting other components that could be identified. Likewise, when fraction IV isolated from aqueous extracts of high-pigmented beetroot powder was subjected to LC-MS analysis, five peaks were identified by combined ultraviolet and MS detection, but *m/z* signals were rather weak and inconclusive.

Fraction I obtained from aqueous extracts of HP beetroot exhibited UV-Vis absorbance maxima of 532 nm, 476 nm, and 320 nm (at relative intensities of 1:4:2), consistent with the presence of betacyanins, betaxanthins, and hydroxycinnamoyl derivatives, respectively (8). This fraction was subjected to LC-MS, and four peaks were identified by combined UV and MS detection. $[M + H]^+$ signals were observed for vulgaxanthin I [*m/z* 340; retention time (RT) = ~5 min], vulgaxanthin II (*m/z* 341; RT = 7.5 min), and (iso)betanin (*m/z* 551; RT = 21–23 and 23–24 min) (Table 2) (39–41). Although betanin and isobetanin can be resolved by LC (39–41), it was not viewed as necessary for our studies, considering that the chemical functionalities of these stereoisomers are essentially identical.

Fraction I originating from the ethanolic extract of Rd beetroot powder was subjected to LC-MS analysis and yielded five to six peaks by combined UV and MS detection. $[M + H]^+$ signals indicated the presence of vulgaxanthin I (*m/z* 340; RT = ~5 min), vulgaxanthin II (*m/z* 341; RT = 7.4 min), and (iso)betanin (*m/z* 551; RT = 22 and 23–24 min). Components eluting at 27–34 min did not exhibit *m/z* signals of known betalains and remain unidentified.

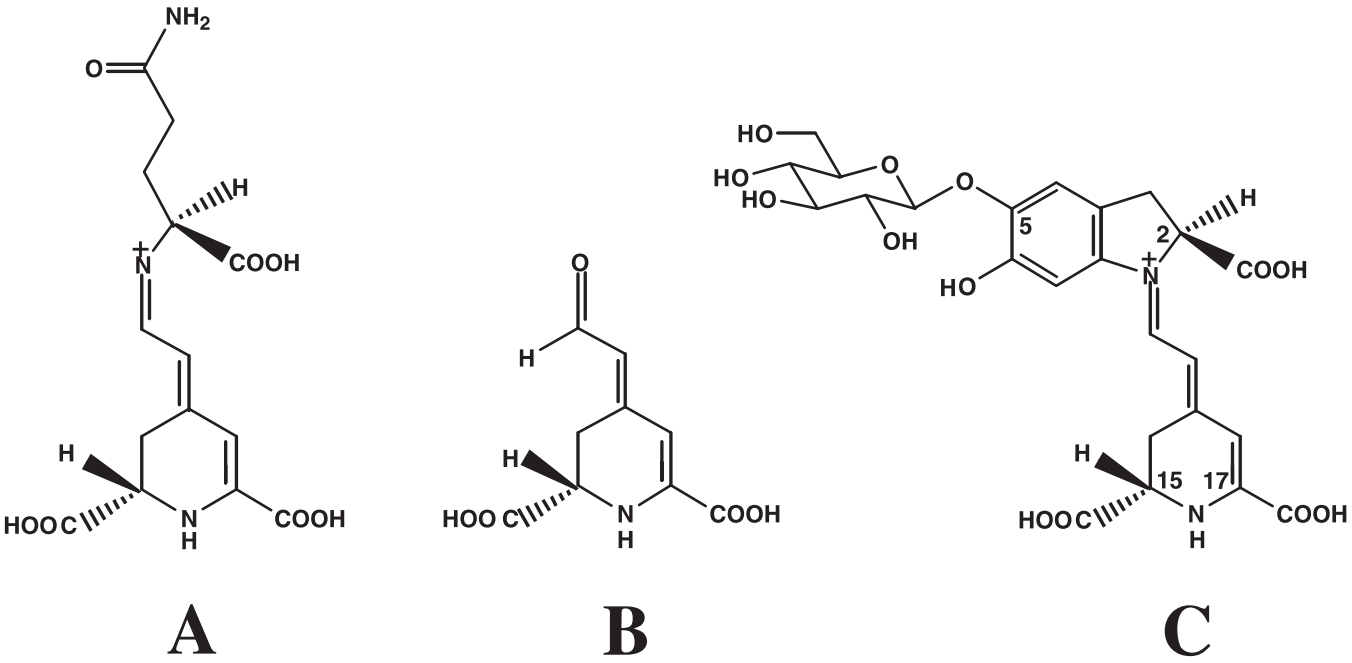

Figure 3. Structures of betalains common to beetroot. A: vulgaxanthin I, B: betalamic acid, and C: betanin.

Table 2. LC-MS Analysis of QR-Inducing Isolates From Beetroot Extracts[a]

Extract and Fraction	LC Retention Time (min)[b]			Tentative Identity[c]
	UV	MS	$[M+H]^+$ (*m/z*)[c]	
Aqueous Red				
Fraction I	Insuff.[d]	Insuff.	Insuff.	
Aqueous High Pigment	5.0	5.1	340	VlgI
Fraction I		7.5	341	VlgII
	21–22	21–22	551	Btn
	23	23–24	551	Btn
EtOH Red	5.0	5.4	340	VlgI
Fraction I		7.4	341	VlgII
	22	22	551	Btn
	23–24	23–24	551	Btn
	27, 31	31, 34	Insuff.	
EtOH High Pigment	4.7	~5	NE[e]	
Fraction I		7.2	NE	
	20–21	20–21	551	Btn
	22–23	22–23	551	Btn
		33 (tr)	NE	
Aqueous High Pigment		5.1	5.2	NE
Fraction IV	7.6	NE		
	11		NE	
	24	24	Insuff.	
		26	Insuff.	
		44	NE	
EtOH Red	5.8	5.8	341	VlgII
Fraction V	19		551	Btn
	22–25	23–24	NE	
	33 (tr)	34 (tr)	NE	

a: Abbreviations are as follows: LC-MS; liquid chromatography-mass spectrometry; QR, quinone reductase; UV, ultraviolet; VlgI, vulgaxanthin I; VlgII, vulgaxanthin II; Btn, betanin and/or isobetanin; NE, not examined.

b: LC profile is listed as retention time as detected by UV or MS analysis.

c: Signals are reported as $[M+H]^+$ values based on known molecular masses of betalains and MS data reported in the literature (8,17,39–41).

d: Insuff., insufficient material or signal was obtained.

Fraction I of ethanolic extracts of HP beetroot powder was subjected to LC-MS, and five peaks were identified by combined UV and MS detection. $[M + H]^+$ signals indicated the presence of (iso)betanin (*m/z* 551; RT = 20–21 and 22–23 min). The peaks eluting at ~5 and 7 min were not analyzed (because they were repeatedly identified, respectively, as vulgaxanthin I and II in other isolates), and there was insufficient material eluting at 33 min to permit analysis.

LC-MS analysis of fraction V isolated from ethanolic extracts of Rd beetroot powder yielded four peaks identified by combined UV and MS detection. The presence of vulgaxanthin II and betanin/isobetanin was indicated by $[M + H]^+$ signals of *m/z* 341 and *m/z* 551 in the first two eluting peaks.

Collectively, all QR-inducing fractions isolated from aqueous and ethanolic extracts contained betalains. The specific betalains routinely identified in these fractions were vulgaxanthin I, vulgaxanthin II (glutamic acid replaces glutamine in Fig. 3A), and (iso)betanin, all of which have been reported as major betalains in beetroots (39,40). The LC order-of-elution patterns observed in this study were identical to those previously reported by others (3,8,17,32), assisting in the identification of specific betalain structures. The dark/black appearance of the active fraction V prepared from HP beets by Sephadex LH-20 chromatography (Table 1) implies that oxidation of betalain pigments may have occurred during extraction and/or isolation. Despite this, *m/z* signals characteristic of betalains were still detected, indicative of the structure of betalains being largely conserved despite the suspected oxidation reactions that may have occurred. For example, if the cyclo-dihydroxyphenylalanine ring of betanin was oxidatively linked through the native 6-OH group, the MS fragmentation pattern may still yield the *m/z* 551 $[M + H]^+$ signal characteristic of (iso)betanin.

It is possible that betalains are only markers for the agents conferring activity in the QR induction bioassay. On occasion, there were unidentified peaks appearing on the high-performance LC (HPLC) chromatograms of samples representing active fractions (Table 2). Aside from these components, there may have been other components in the isolates that escaped detection because the selected HPLC protocol did not resolve or elute them. Recent studies have detected unique ferulic acid derivatives in beetroot tissues (3,13,17,39,40), and ferulic acid is an effective phase II enzyme inducer in vivo (37). Nevertheless, we provided evidence that betalains, and, more specifically, betacyanins (red/purple betalains), may at least be biomarkers for the active agents if they are not the active agents themselves. Our previous report that phase II enzyme-inducing activity could only be observed in extracts of tissues from beetroots of red but not orange or white phenotypes (20) infers that betacyanins and not betaxanthins are associated with in vitro QR-inducing activity.

Animal Feeding Trials

The levels of beetroot isolate used in the experimental diets were limited to 10 ppm and 150 ppm, in part based on the scale-up requirements of preparing the isolates. Despite this constraint, the levels of isolate included in the diets were calculated to be about equivalent to a human intake of about one beet per day. This calculation is based on yields of extract and isolate and extrapolation from rodent to human dietary intake. Consequently, the reader is cautioned that such calculations are rather coarse and should not be considered authoritative. Although animal trials using potent phase II enzyme inducers such as olpitraz (42), or copious quantities of preparations that are suspected to possess inducing power (43), have made use of short dietary interventions of ≤1 wk, our study employed a longer-term trial because of the low level of supplementation of partially purified isolates of modest in vitro activity. Longer-term dietary trials for phase II enzyme induction over 4–5 wk have also been employed for other crude preparations such as green tea polyphenols (44) and garlic powder (45).

Statistical analysis of the tissue levels of QR and GST in the five organs isolated from animals subject to dietary intervention trials revealed no significant effect of diet (Table 3). However, there was a significant effect of time on tissue levels of some of these enzymes in various tissues, and this influence may be founded on animal developmental factors. No significant differences in average weight gains (calculated as g day^{-1}) among animals were observed for control, diet group A, and diet group B at 2.51 ± 0.50, 2.70 ± 0.35, and 2.72 ± 0.33 over the 1st mo, respectively, and 2.46 ± 0.27, 2.61 ± 0.45, and 2.43 ± 0.34 over the entire 2-mo study, respectively.

There was no significant in vivo phase II enzyme-inducing effect overall of betalain-supplemented diets, but a significant time effect was observed (Table 3, Fig. 4). There was also a broad range of responses of individual animals. Two of the animals from diet group B exhibited the greatest levels of QR-specific activity in intestinal tissue among their group/time cell ($n = 8$) at 30 days (Fig. 4C). These same animals exhibited the two greatest QR-specific activities for colon, kidney, and lung tissue as well (Fig. 4A,E, and I). In addition, one of these animals exhibited the greatest values recorded for GST-specific activity among members of this group/time cell for colon, intestinal, and lung tissue (Fig. 4B, D, and J). Similarly, one animal of the control group exhibited the greatest levels of QR-specific activity among members of this group at 30 days for colon, intestinal, kidney, and lung tissues (Fig. 4A, C, E, and I). Levels of QR and GST in tissues of this same animal for five of the other six enzyme/tissue combinations measured were among the top two values recorded among members of this sampling cell ($n = 8$).

The broad range of biochemical responses of rats to betalain-supplemented diets was not surprising given the known disparity in responses of humans to ingested betalain pigments. As much as 15% of the human population is subject to an innocuous condition of "beeturia", the symptoms of which are pink- or red-colored urine following ingestion of Rd beetroot or extract (46). The susceptibility of individuals to beeturia has been attributed to genetic factors, allergies, and iron maladsorption. On the other hand, the extent to

Table 3. Summary of Analyses for Quinone Reductase and Glutathione *S*-Transferase Activities in Tissues of Rats Subject to Experimental Diets[a]

Organ and Enzyme	Enzyme Activities Observed Within All Dietary Groups at		
	0 days (n = 8)	30 days (n = 24)	60 days (n = 27)
Colon			
QR*[b] (0.467)[c]	16.4 ± 1.2 (16.3)	25.9 ± 9.4 (24.8)	36.8 ± 7.4 (38.8)
GST* (0.529)	8.3 ± 0.6 (8.2)	15.4 ± 1.4 (15.6)	16.2 ± 2.3 (16.1)
Intestine			
QR* (0.301)	16.5 ± 3.5 (15.3)	51.4 ± 25.3 (48.6)	24.4 ± 8.9 (22.6)
GST* (0.651)	39.2 ± 10.8 (38.8)	102.2 ± 32.0 (109.2)	48.8 ± 19.2 (49.5)
Kidney			
QR* (0.307)	17.7 ± 2.5 (18.2)	14.3 ± 2.2 (14.0)	12.2 ± 1.9 (11.8)
GST (0.859)	18.9 ± 2.5 (18.5)	17.3 ± 6.7 (17.0)	16.7 ± 2.2 (17.1)
Lung			
QR* (0.856)	76.2 ± 5.0 (75.8)	48.8 ± 6.8 (46.7)	51.3 ± 3.9 (51.6)
GST* (0.382)	21.0 ± 1.1 (21.0)	16.8 ± 2.8 (16.0)	21.4 ± 1.8 (21.4)
Liver			
QR (0.569)	38.4 ± 7.7 (35.4)	41.8 ± 11.8 (41.6)	38.1 ± 7.6 (35.4)
GST* (0.764)	101.7 ± 11.3 (103.7)	115.4 ± 23.1 (112.2)	85.2 ± 9.8 (85.5)

a: Abbreviations are as follows: QR, quinone reductase; GST, glutathione *S*-transferase. Enzyme activities are expressed as units (μmol/min) per milligram of protein and reported as group mean ± SD, with group median values in parentheses.

b: $*P < 0.001$ for effect of time.

c: Numbers in parentheses represents *P* values derived from statistical analysis of effect of treatment on measured parameter.

which individuals absorb and excrete betalains may be simply related to dynamics of digestion, specifically, variations in gastric pH and emptying rate (47). In any event, the range in bioavailability among individuals (human or animal) may prohibit any clear and statistically significant demonstration on pharmacological effect of betalains in virtually any feeding trial.

The recovery of intact betalains excreted in the urine of 100 "normal (human) subjects", defined as those free of obvious or documented medical problems, ranged from 0.01 to 0.6% (47). More recent studies employing human subjects yielded estimates of 0.5–0.9% (11) and 3.0–3.7% (48). These should be considered minimal estimates because some bioavailable betalains may be transformed during metabolism. In any event, for the 10–15% of the human population that are "absorbers" of betalains, an in vivo effect on inducing phase II enzymes may be possible. If individual rats can also be differentiated as (non)absorbers, then the animals in the present study fed the betalain-supplemented diet and consistently exhibiting the greatest tissue levels of QR and GST may represent the absorber group. The only other reports we could locate on a cancer-chemopreventive benefit of betalains in animals made use of a crude beetroot extract where no measure was used to demonstrate that the effect of the crude extract could be attributed directly to the betalains (14,49).

Betanin as an In Vitro Quinone Reductase Inducer From Beetroot Extracts

Subsequent to the animal studies and initial review of this article, we sought to definitively test if betalains, specifically, the red betacyanins as implicated previously (20), were in vitro QR inducers. A crude aqueous extract of HP beetroots (~1 g dry matter) was separated by Sephadex LH-20, yielding five distinct visually colored "betalain" fractions. The nature and recovery of the fractions of particular interest in order of elution were I, brown, 30 mg; II, light red, 10 mg; and III, purple, 820 mg. Other fractions collected in limited quantities and of less further interest (because of relative lack of betacyanins) included fractions IV (orange), V (pink), and VI and VII (both clear). All seven fractions were lyophilized and tested in the QR induction bioassay, and only betalain fractions I and III were capable of significant QR induction in vitro (Fig. 5A). Although fraction I exhibited greatest potency of QR induction, betalain fraction III was subject to further purification because it was the most abundant betalain fraction.

Betalain fraction III was resolved by preparative LC into three purified components, each of which was examined in the Prochaska bioassay (Fig. 5B) and subjected to ESI-MS and ESI-MS-MS analysis (Table 4). Betalain isolate IIIa was a weak QR inducer, with significant induction occurring only at 0.25 mg/ml. This component remains unidentified because the MS and MS-MS data are not representative of known betalains, even though the absorbance at 480 nm is a general property of betaxanthins. Betalain isolate IIIb was capable of doubling QR-specific activity in the bioassay at 0.12 mg/ml (Fig. 5B), and MS data confirmed the identity of this isolate as (iso)betanin based on the *m/z* signal of 551 (Table 4). The low-intensity *m/z* signals of 507 and 505 correspond to the decarboxylated and the decarboxylated and dehydrogenated (iso)betanin derivatives, respectively, which are known to form in thermally processed beet juice (50). These same derivatives could also be formed under the conditions of ESI-MS analysis of betanin. The MS-MS analyses of the *m/z*

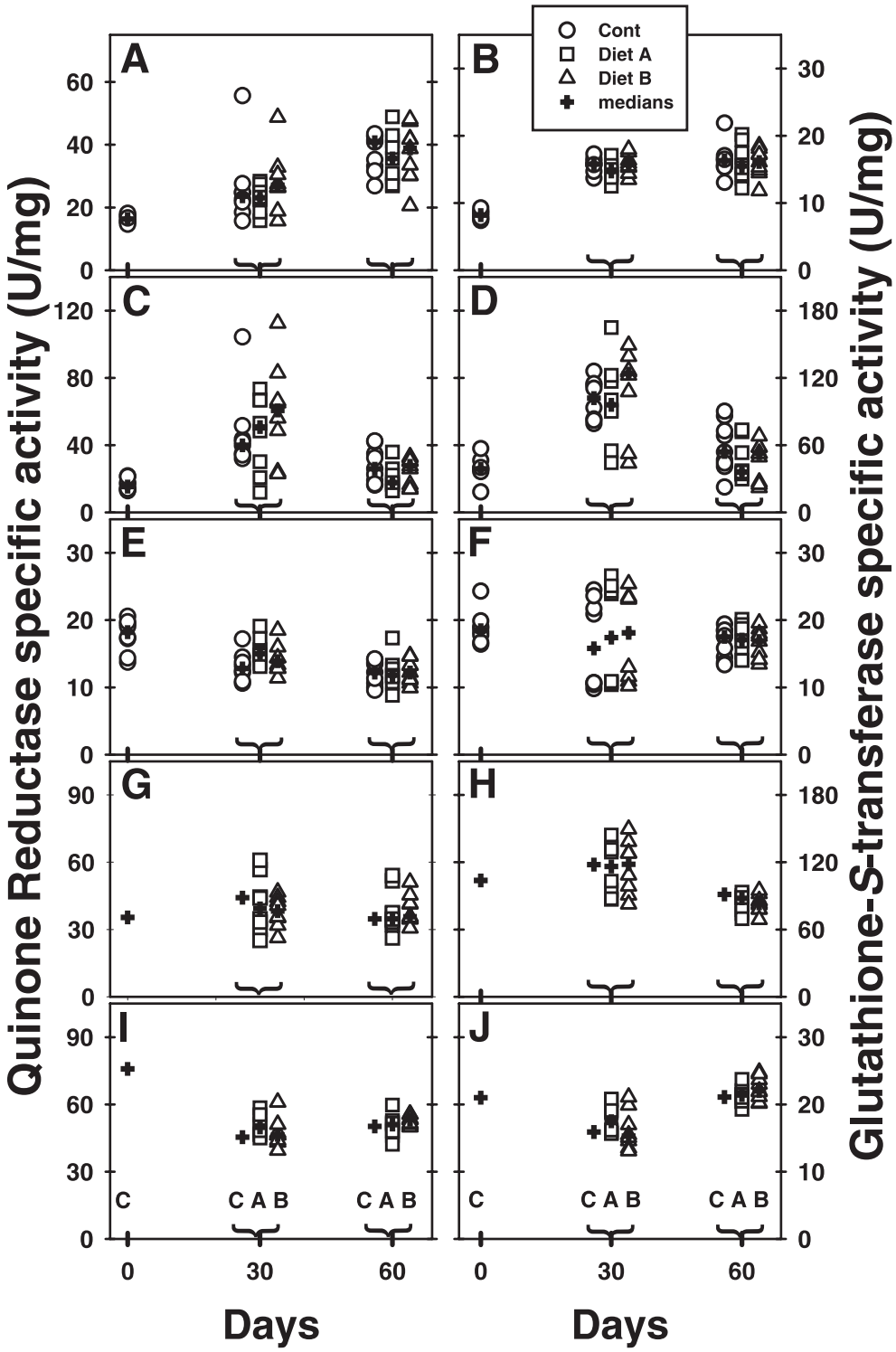

Figure 4. Effects of betalain-supplemented diets on rat tissue levels of phase II enzymes. Specific activities of quinone reductase are shown for colon (A), intestinal (C), kidney (E), liver (G), and lung (I) tissues; specific activities of glutathione *S*-transferase are shown for colon (B), intestinal (D), kidney (F), liver (H), and lung (J) tissues. Day 0 ($n = 8$) represents animals conditioned on the control diet (C) for 2 wk prior to being placed on betalain-supplemented diets. At intervals of 30 ($n = 8$) and 60 ($n = 9$) days, responses are grouped as columns from left to right, representing "control" (C, open circles), "diet A" (A, open squares), and "diet B" (B, open triangles) groups, respectively. Median values for each treatment group at each time interval are displayed as a cross among the data each set.

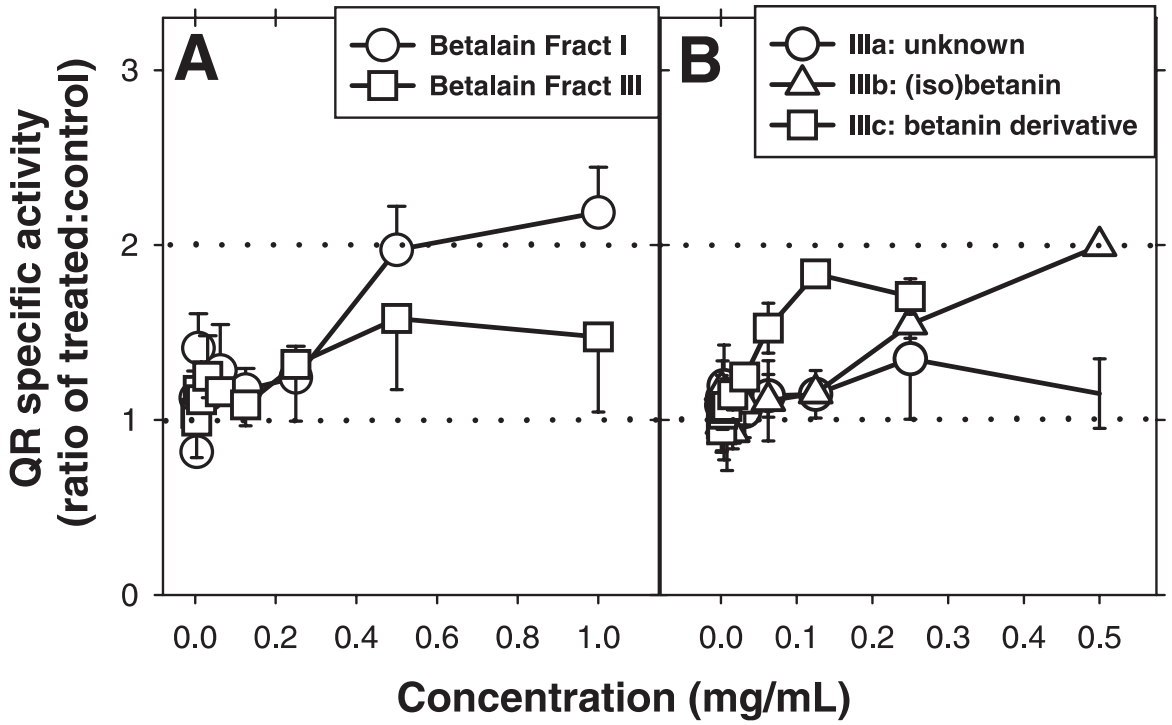

Figure 5. A: Quinone reductase partially purified betalain fractions. B: Purified components from betalain fraction III. Results are expressed as mean ± SD from four determinations. Dose-dependent responses for all isolates shown were different ($P < 0.05$) from noninduced controls.

Table 4. Properties of Purified Components Resolved From Betalain Fraction III by Preparative Liquid Chromatography[a]

Isolate (retention time by LC)	Absorbance[b] 480 nm	Absorbance[b] 532 nm	Mass Spectrometry Data[c]	Identity
IIIa (4.2 min)	0.742	~0	365 (100), 215 (30), 707 (18), 460 (16), 489 (14), 533 (9) MS^2 [707]: 365 (100)	Unknown betaxanthin (tentative)
IIIb (17.3 min)	0.718	1.820	551 (100), 507 (4), 505 (4) MS^2 [551]: 389 (100) MS^2 [507]: 345 (100) MS^2 [505]: 343 (100)	(Iso)betanin
IIIc (27.9 min)	0.061	0.158	340 (100), 365 (80), 551 (67), 216 (61), 409 (44), 505 (24), 314 (23), 722 (6) MS^2 [340]: 323 (100), 277 (10) MS^2 [551]: 389 (100) MS^2 [409]: 216 (100), 391 (32), 289 (20) MS^2 [722]: 243 (100), 409 (6)	(Iso)betanin derivative (tentative)

a: Abbreviations are as follows: LC, liquid chromatography; MS, mass spectrometry.
b: Peak absorbance units during LC analysis of isolate.
c: *m/z* $[M + H]^+$ values listed in decreasing order of intensity (in parentheses). MS^2 (MS-MS) analysis was conducted on the *m/z* $[M + H]^+$ signal indicated in brackets.

fragments 551, 507, and 505 were also conclusive evidence of the original (iso)betanin structure and corresponding loss of the glucosyl moiety with a reduction in *m/z* signal of 162 to yield the (iso)betanidin unit. Lastly, the ratio of absorbance at 532 nm/480 nm of ~2.5 is almost identical to that of pure betanin (51).

Betalain isolate IIIc significantly induced QR in vitro at levels as low as 0.031 mg/ml in the bioassay (Fig. 5B). MS analysis indicated the presence of both vulgaxanthin I (*m/z* 340) and (iso)betanin (*m/z* 551), with the latter in greater abundance based on the absorbance ratio at 532 nm/480 nm of ~2.5, and observance of a dominant red band on thin-layer

chromatography (TLC) coinciding with the migration of pure (iso)betanin obtained as betalain isolate IIIb (data not shown). The *m/z* signal of 314 $[M + H]^+$ is evidence of *N-trans*-feruloyltyramine, a phenolic amide known to exist in beetroot (40). The observation that these *m/z* $[M + H]^+$ signals, corresponding to (iso)betanin, vulgaxanthin I, and *N-trans*-feruloyltyramine, were obtained in a purified isolate that exhibited reasonable peak symmetry on analytical and preparative LC implies the existence of a specific chemical species where these structures are covalently linked but remain intact. The nature of this isolate remains to be identified.

Conclusions

The components of beetroot extracts capable of inducing phase II enzyme activities in Hepa 1c1c7 cells were confirmed to be betalains, although components that co-purify with betalains may also contribute to this activity. Only extracts of red/purple-pigmented beetroot tissues were capable of QR induction in vitro, whereas extracts of beetroot tissues diminished or lacking in betacyanins were not (20). A seminal study demonstrated acetonitrile extracts of beetroot tissue to be ineffective at inducing QR in the Hepa 1c1c7 bioassay (52). Because betalains are essentially insoluble in acetonitrile (20), this further implicates betacyanins as the predominant QR-inducing agents in beetroot extracts. Beetroot is also rich in ferulic acid derivatives (3,15–17,39,40), and such compounds may also mediate QR induction (53). Although the rodent feeding trials using diets supplemented with low doses of betalain-enriched isolates did not show in vivo efficacy, the subsequent revelation that (iso)betanin is likely the primary QR inducer in beetroot extracts provides encouragement for a follow-up animal trial making use of more enriched betalain-supplemented diets. Betalains have recently been shown to be at least 3–4% bioavailable in humans and associated with a reduced sensitivity of low-density lipoprotein (LDL) to ex vivo oxidation (48).

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