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Antioxidant Activity of Betalains from Plants of the Amaranthaceae

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Antioxidant activity of betalain pigments (seven pure compounds and four combined fractions) from plants of the family Amaranthaceae was evaluated using the modified DPPH• (1,1-diphenyl-2-picrylhydrazyl) method. All tested betalains exhibited strong antioxidant activity. Their EC_{50} values ranged from 3.4 to 8.4 μM. Gomphrenin type betacyanins (mean = 3.7 μM) and betaxanthins (mean = 4.2 μM) demonstrated the strongest antioxidant activity, 3−4-fold stronger than ascorbic acid (13.9 μM) and also stronger than rutin (6.1 μM) and catechin (7.2 μM). Antioxidant activity of the tested betalains decreased in the following order: simple gomphrenins > acylated gomphrenins > dopamine-betaxanthin > (*S*)-tryptophan-betaxanthin > 3-methoxytyramine-betaxanthin > betanin/isobetanin > celosianins > iresinins > amaranthine/isoamaranthine. This study also investigated and discussed the relationship between the chemical structure and the activity of the betalains. The free radical scavenging activity of the betalains usually increased with the numbers of hydroxyl/imino groups and, moreover, depended on the position of hydroxyl groups and glycosylation of aglycones in the betalain molecules.

KEYWORDS: Antioxidant activity; Amaranthaceae; betalains; betacyanins; betaxanthins; DPPH•; antioxidants; colorants

INTRODUCTION

There is currently considerable interest in dietary antioxidants as bioactive components of food. There is also evidence suggesting that some natural colorants may be nutritionally important antioxidants and that their presence in the diet may reduce the risk of cardiovascular disease, cancer, and other diseases associated with aging (*1*−*4*). Most research has been focused on natural colorants such as carotenoids, anthocyanins (flavonoids), and curcuminoids, which exhibit antioxidant, antiinflammatory, and antibacterial (antiviral) activities and anticarcinogenic and antitumor-promoting effects (*5*−*10*).

Betalains from red beet have been extensively used in the modern food industry. The betalains are one of the most important natural colorants and are also one of the earliest natural colorants developed for use in food systems (*11*). However, little is known about the health effects of betalains as compared to other natural colorants, such as carotenoids and anthocyanins. Recently, several studies on the antiradical and antioxidant activity of betalains (mainly betanin) from beet roots (*Beta vulgaris*) have been published (*12*−*15*). These results demonstrated that the betalains from beet roots possessed high antiradical effect and antioxidant activity, representing a new class of dietary cationized antioxidants. Betanin at very low concentrations was found to inhibit lipid peroxidation and heme decomposition. The IC_{50} inhibition concentration for low-density lipoprotein (LDL) oxidation (by H_2O_2-activated metmyoglobin) of betanin was better than that of catechin. Red beet products used regularly in the diet may provide protection against certain oxidative stress-related disorders in humans (*15*).

Since 1996, we have been conducting research on the development of betalains from plants of the family Amaranthaceae as natural colorants. The betacyanins from *Amaranthus* and the betaxanthins from *Celosia* exhibit bright red-violet or yellow color characteristics and favorable stability and can be applied in some food systems; furthermore, their plant materials have high biomass (*16*, *17*). The results suggest that these betalains have high potential not only as natural food colorants but also as a substitute source for the well-known betalains from red beets.

A total of 16 red-violet betacyanins and three yellow betaxanthins have been separated and identified from plants of the Amaranthaceae family in our earlier studies (*18*−*20*). Previous studies (*12*, *13*, *15*) on antioxidant activity were restricted to only a few betalains from beet roots (*B. vulgaris*) but not from the Amaranthaceae plants (except *Amaranthus tricolor*), which contain a great number of different betalains. Preliminary data revealed that there were marked differences in antioxidant activity among several betalains (betanin, amaranthine, and vulgaxanthin I and II).

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Table 1. Antioxidant Activities of Various Betalains Separated from Plants of the Amaranthaceae by Preparative HPLC

compds	collected peak[a]	EC_{50} (μM)[b]	inhibition (5 μM) (%)[c]	antiradical activity (initial slope)[d]	plant species or source
		red-violet betacyanins			
amaranthine type betacyanins					
amaranthine	1	8.37	31.1 ± 0.16	−3.14 ± 0.08	*A. tricolor*
isoamaranthine	1′	8.35	31.1 ± 0.20	−3.14 ± 0.06	*A. tricolor*
iresinins (acylated amaranthine)	3, 3′	8.08	32.4 ± 0.12	−3.20 ± 0.16	*I. herbstii*
celosianins (acylated amaranthine)	4, 4′, 5, 5′	7.13	34.6 ± 0.07	−3.55 ± 0.14	*C. cristata* (violet)
betanin type betacyanins					
betanin	2	4.88	50.8 ± 0.12	−5.28 ± 0.08	red beet powder
isobetanin	2′	4.89	50.8 ± 0.24	−5.27 ± 0.11	red beet powder
gomphrenin type betacyanins					
(iso)gomphrenin I	6, 6′	3.35	74.5 ± 0.17	−8.18 ± 0.13	*G. globosa*
acylated gomphrenins	7, 7′, 8, 8′	4.11	59.9 ± 0.11	−6.37 ± 0.05	*G. globosa*
		yellow betaxanthins			
dopamine-betaxanthin	9	4.08	60.3 ± 0.25	−6.32 ± 0.05	*C. plumosa* (yellow)
3-methoxytyramine-betaxanthin	10	4.21	56.9 ± 0.08	−5.71 ± 0.07	*C. plumosa* (yellow)
(*S*)-tryptophan-betaxanthin[e]	11		53.6 ± 0.27		*C. plumosa* (yellow)
		standard antioxidants			
ascorbic acid		13.93	17.1 ± 0.08	−2.03 ± 0.03	BDH
rutin (flavonol)		6.11	40.9 ± 0.22	−4.09 ± 0.12	Sigma
(+)-catechin (flavanol)		7.24	33.7 ± 0.34	−3.28 ± 0.05	Sigma
ferulic acid (phenolics)		19.35	14.7 ± 0.15	−1.27 ± 0.09	Sigma
$LSD_{0.05}$[f]			2.3	−0.33	

[a] Peak numbers coincide with the numbers for betalains in **Figure 1**. [b] EC_{50} values were calculated from the slope equations of the dose–response curves (r^2 = 0.967–0.999). [c] Values were means of inhibition (%) of DPPH• at 5 μM antioxidants ± standard derivation (SD) (n = 3). [d] Values were means of slope coefficients by linear regression (r^2 = 0.935–0.992) ± SD (n = 3) in μM DPPH•/μM test samples. [e] The amount of the collected sample (peak 11) was very low, so it was only used for measurement of inhibition (%). [f] Least significant difference for comparison of betalain means ($p < 0.05$).

Two free radicals that have been used for assessing antioxidant activity are 2,2′-azino-bis-(3-ethyl-benzthiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) and 1,1-diphenyl-2-picrylhydrazyl (DPPH•) (*21*, *22*). Brand-Williams et al. (*23*) established the DPPH• method. Reduction of DPPH• by an antioxidant (A) (DPPH• + A → DPPH−H + A•) or by a radical species (R) (DPPH• + R• → DPPH−R) causes a loss of absorbance at 515 nm. Miller et al. (*24*) and Miller and Rice-Evans (*25*) developed the $ABTS^{\bullet+}$ method (Trolox equivalent antioxidant activity, absorbance at 734 nm). Escribano et al. (*12*) modified the $ABTS^{\bullet+}$ method to measure antiradical activity of betalains by the disappearance of $ABTS^{\bullet+}$ radical at 414 nm. However, the test procedure seemed to be complicated. Before assay, it took more time to generate $ABTS^{\bullet+}$ radical in a complex reaction medium containing 2,2′-azino-bis-(3-ethyl-benzthiazoline-6-sulfonic acid) diammonium salt (ABTS), H_2O_2, horseradish peroxidase, and sodium acetate (*14*). Moreover, it was not easy to control $ABTS^{\bullet+}$ concentration. The DPPH• method is one of the simplest assay methods and has commonly been used for assessing antioxidant activity of natural colorants (anthocyanins and carotenoids) and phenolic compounds (*9*, *22*, *26*). To date, the suitability of the DPPH• method has not been established for testing antioxidant activity of betalains.

The objective of the present work was (i) to determine and evaluate antioxidant activity of different betalains from the plants of the Amaranthaceae as a potential source of both natural antioxidants and natural colorants and (ii) to investigate the relationship between the chemical structure and the activity of the betalains.

MATERIALS AND METHODS

Materials. Betalain-containing plant materials (**Table 1**) were selected from 37 species of eight genera of the family Amaranthaceae and were grown in Wuhan, China, as recently described (*17*, *19*). Samples of fresh materials (inflorescences and leaves) were harvested, frozen, and kept at −18 °C until use.

DPPH•, rutin, catechin, and ferulic acid were purchased from Sigma Chemical Co. (St. Louis, MO). L-Ascorbic acid, formic acid, and other solvents (methanol and ethanol) were obtained from BDH Laboratory Supplies (Poole, England).

Extraction and Purification of Betalains. Betalains were extracted from frozen plant materials with 80% methanol and purified as described (*17*, *19*) with some modification. The betalain extracts were filtered by a Millipore filter (Millipore Corp., Bedford, MA) with a 0.2 μM nylon membrane under vacuum at 22 °C and centrifuged at 20 000*g* for 20 min at 4 °C. The supernatant was concentrated under vacuum and then transferred to a 100 cm × 2.5 cm i.d. Sephadex LH-20-100 column and separated by elution with ultrafiltered water adjusted to pH 5−6 with formic acid. Different fractions (red-violet betacyanins and yellow betaxanthins) were collected and then freeze-dried in a Heto FD3 freeze-dryer (Heto-Holten A/S, Denmark) to obtain partially purified betalain powders. The final purification was carried out by preparative high-performance liquid chromatography (HPLC) with different chromatographic conditions. Peak collection for different betalains is shown in **Table 1** and described in the next section. The completely purified betalain samples were freeze-dried again and used for the antioxidant activity assay.

HPLC. Preparative and analytical HPLC were performed with a Hewlett-Packard HPLC System (HP 1100 series, Waldbronn, Germany) operated at room temperature (22 °C). The liquid chromatograph was equipped with a 250 mm × 9.4 mm i.d., 5 μm, Zorbax SB-C18 column (Agilent Technologies, U.S.A.) for preparative HPLC or with a 250 mm × 4 mm i.d., 5 μm, Nucleosil C18 column (Agilent Technologies) for analytical HPLC. Conditions for preparative HPLC were as follows: solvent A was 1% aqueous formic acid, and solvent B was 80% aqueous MeOH. Different linear gradients were used as follows: (i) amaranthines: 0−17 min, 10−45% B; 17−21 min, 45−100% B; (ii) iresinins: 0−25 min, 15−60% B; 25−35 min, 60−100% B; (iii) celosianins: 0−28 min, 20−70% B; 28−35 min, 70−100% B; (iv) betanins: 0−25 min, 10−55% B; (v) gomphrenins: 0−40 min, 20−100% S; and (vi) betaxanthins: 0−25 min, 20−70% B; 25−35 min, 70−100% B. The injection volume was 100 μL, and a flow rate of 3.5 mL/min was used. The separations were monitored at 538 or 540 nm for betacyanins and at 474 nm for betaxanthins. Flavonoids were

monitored at 360 nm and phenolic acids at 280 nm. Conditions for analytical HPLC of betalains were previously described (*17*, *19*).

Assessment of Antioxidant Activity. Modifications were made to the original DPPH• method described by Brand-Williams (*23*). Both DPPH• and the test compounds were prepared in 80% ethanol instead of methanol, according to Son and Lewis (*27*). DPPH• solution (60 μM, 3.9 mL) was added to 0.1 mL of the test compounds at different concentrations. At least six different concentrations were used. The final concentrations of the test compounds in the reaction mixtures were 0.25−10 μM for all betalain samples and two standard samples (rutin and catechin) and 1.5−25 μM for other samples (ascorbic acid and ferulic acid). The reaction for scavenging DPPH• radicals was in 15 mL polypropylene conical tubes (Becton Dickinson, NJ) at room temperature (22 °C). The decrease in absorbance of DPPH• at 515 nm was measured at different time intervals by a Spectronic Genesys 5 spectrophotometer (Milton Roy, NY) until the reaction reached a plateau. Most tested compounds reacted completely within 180 min in the conditions of this study, and ascorbic acid reacted within 1 min. Ethanol (80%) was used as a blank solution, and DPPH• solution without test samples (3.9 mL of DPPH• + 0.1 mL of 80% ethanol) served as the control. All tests were performed in triplicate. Antioxidant activity of the test samples was expressed using the parameters of (i) radical scavenging activity: EC_{50} = concentration (μM) of antioxidant (test sample) required for a 50% decrease in absorbance of DPPH• radicals; (ii) inhibition (%) of DPPH• absorbance = $(A_{control} - A_{test}) \times 100/A_{control}$ (*27*). A plot of absorbance of DPPH• vs concentration of antioxidant was made to establish the standard curves (dose−response curves) and to calculate EC_{50}. $A_{control}$ is the absorbance of the control (DPPH• solution without test sample), and A_{test} is the absorbance of the test sample (DPPH• solution plus 0.1 mL of 5 μM test compound). According to the modified DPPH• method of Fukumoto and Mazza (*22*), antioxidant activity was expressed by antiradical activity, which was defined as the initial slope values on the plot of concentration (μM) of the test sample vs concentration (μM) of DPPH• in the final reaction time. The reaction kinetic behavior was reflected by the plot of each concentration of the test sample vs the percentage of remaining DPPH• for each measured time interval. The DPPH• concentration ($C_{DPPH•}$) (μM) in the reaction medium (80% ethanol) was calculated from the following calibration curve determined by linear regression: $A_{515nm} = 0.0113 \times C_{DPPH•} + 0.0027$ ($r^2 = 0.999$). The concentration (μM) of the test betalains was from the corresponding molar absorptivity (ϵ). The mean ϵ value for betaxanthins is 4.80×10^4 (*20*, *28*); for amaranthine type betacyanins, it is 5.66×10^4 (*18*, *29*); for betanin type betacyanins, it is 6.16×10^4 (*19*, *30*); and for gomphrenin type betacyanins, it is 5.06×10^4 (*19*, *31*).

RESULTS AND DISCUSSION

Preparation of Purified Betalains. Standard samples of betalains are not currently commercially available. It is important to separate and purify betalain samples sufficiently for accurate analysis of their antioxidant activity. It is laborious and time-consuming to obtain sufficient quantities of the different pure betalains. Crude betalain extracts usually contain some phenolic acids and flavonoids, similar to betalains in molecular weight and in some properties. Phenolic acids and flavonoids themselves generally have antioxidant activity, so if they are not completely removed from betalain extracts, they will interfere with accurate measurement of antioxidant activity of the betalains. In previous studies (*12*, *15*), betalains (four types) from beet roots for evaluation of antioxidant activity were prepared only by gel filtration chromatography (Sephadex G-25 column), making it difficult to obtain pure samples. In this study, 16 red-violet betacyanins (amaranthine, betanin, celosianin I, celosianin II, gomphrenin I, gomphrenin II, gomphrenin III, iresinin I, and their corresponding stereoisomers at C-15) and three yellow betaxanthins (dopamine-betaxanthin, 3-methoxytyramine-betaxanthin, and (*S*)-tryptophan-betaxanthin) were obtained from selected different species of the family Amaranthaceae by gel filtration chromatography (Sephadex LH-20-100 column) and preparative HPLC (Zorbax SB-C18 column) (**Figure 1** and **Table 1**). Identification of these separated peaks can be easily ascertained from our previous data (e.g., analytical HPLC and LC-MS) and reference samples (*17*, *19*, *20*). **Figure 1** shows preparative HPLC profiles of the 19 betalains, which are similar to the analytical HPLC profiles. The 16 types of betacyanins were divided into three types, i.e., amaranthine type, betanin type, and gomphrenin type betacyanins (*19*) (**Table 1** and **Figure 1**). Their chemical structures are also summarized in **Figure 1**. Celosianins I and II, iresinin I, and their C-15 epimers were acylated amaranthines, identified as betanidin 5-*O*-β-glucuronosylglucoside acylated with ferulic, *p*-coumaric, or 3-hydroxy-3-methylglutaric acids. Gomphrenins II and III and their C-15 epimers were acylated gomphrenins, identified as betanidin 6-*O*-β-glucoside acylated with ferulic and *p*-coumaric acid. The number of betalains in this paper coincides with the number of peaks in **Figure 1** and **Table 1**.

A suitable amount of pure amaranthine (**1**) and isoamaranthine (**1′**) (**Figure 1A**) was readily isolated from *A. tricolor* leaves. Betanin and isobetanin were also isolated from many *Amaranthus* genotypes previously described (*19*), but their content was quite low, and the quantity of collected pure samples was not sufficient for the antioxidant activity assay. Therefore, pure betanin (**2**) and isobetanin (**2′**) (**Figure 1D**) were separated from red beet powder (No. 3600, Warner-Jenkinson Company, Inc., St. Louis, MO) in this study. Amaranthine/isoamaranthine or betanin/isobetanin had similar structures and similar antioxidant activities (**Table 1**), suggesting that other betalain compounds and their corresponding C-15 epimers should have similar antioxidant activities. Because the C-15 epimer (isoiresinin I, **3′**) of iresinin I (**3**) from *Iresine herbstii* leaves occurred at very low concentrations, pure iresinins were collected together from the peaks for **3** and **3′** (**Figure 1B** and **Table 1**). Considering the difficulty of collecting sufficient pure celosianins and gomphrenins, it was necessary to combine the betacyanins with similar structures. Pure celosianins consisted of four peaks, i.e., celosianin I (**4**), celosianin II (**5**), and their C-15 epimers (**4′**, **5′**) isolated from *Celosia cristata* inflorescences (**Figure 1C** and **Table 1**). Pure nonacylated gomphrenins were collected from the peaks for **6** (gomphrenin I) and **6′** (isogomphrenin I), while pure acylated gomphrenins were combinations of the peaks for **7**/**7′** (gomphrenin II/isogomphrenin II) and peaks **8**/**8′** (gomphrenin III/isogomphrenin III) isolated from *Gomphrena globosa* inflorescences (**Figure 1E** and **Table 1**). Three pure betaxanthins were collected from the peaks for **9**−**11**, respectively (**Figure 1F**), isolated from yellow inflorescences of *Celosia plumosa*. Because the content of (*S*)-tryptophan-betaxanthin (**11**) was very low, the collected sample was used only for assay of inhibition (%).

Antioxidant Activity. Table 1 lists the antioxidant activity of the purified betalains and some standard compounds. EC_{50} is a parameter widely used to express antioxidative power (*26*, *32*, *33*). The lower the EC_{50} value, the higher the potential antioxidant activity. From **Table 1**, all betalain compounds exhibited stronger antioxidative power (EC_{50} = 3.35−8.37 μM, mean = 5.75 μM) than ascorbic acid (13.93 μM), a common antioxidant. Of the betalains studied, gomphrenin type betacyanins (mean = 3.73 μM) and betaxanthins (mean = 4.15 μM) demonstrated the strongest antioxidant activity, 3−4-fold stronger than ascorbic acid. As compared to typical natural antioxidants (rutin and catechin) (EC_{50} = 6.11 and 7.24 μM), most betalain compounds also exhibited higher antioxidant activity, except iresinins (8.08 μM) and amaranthine/isoamaranthine

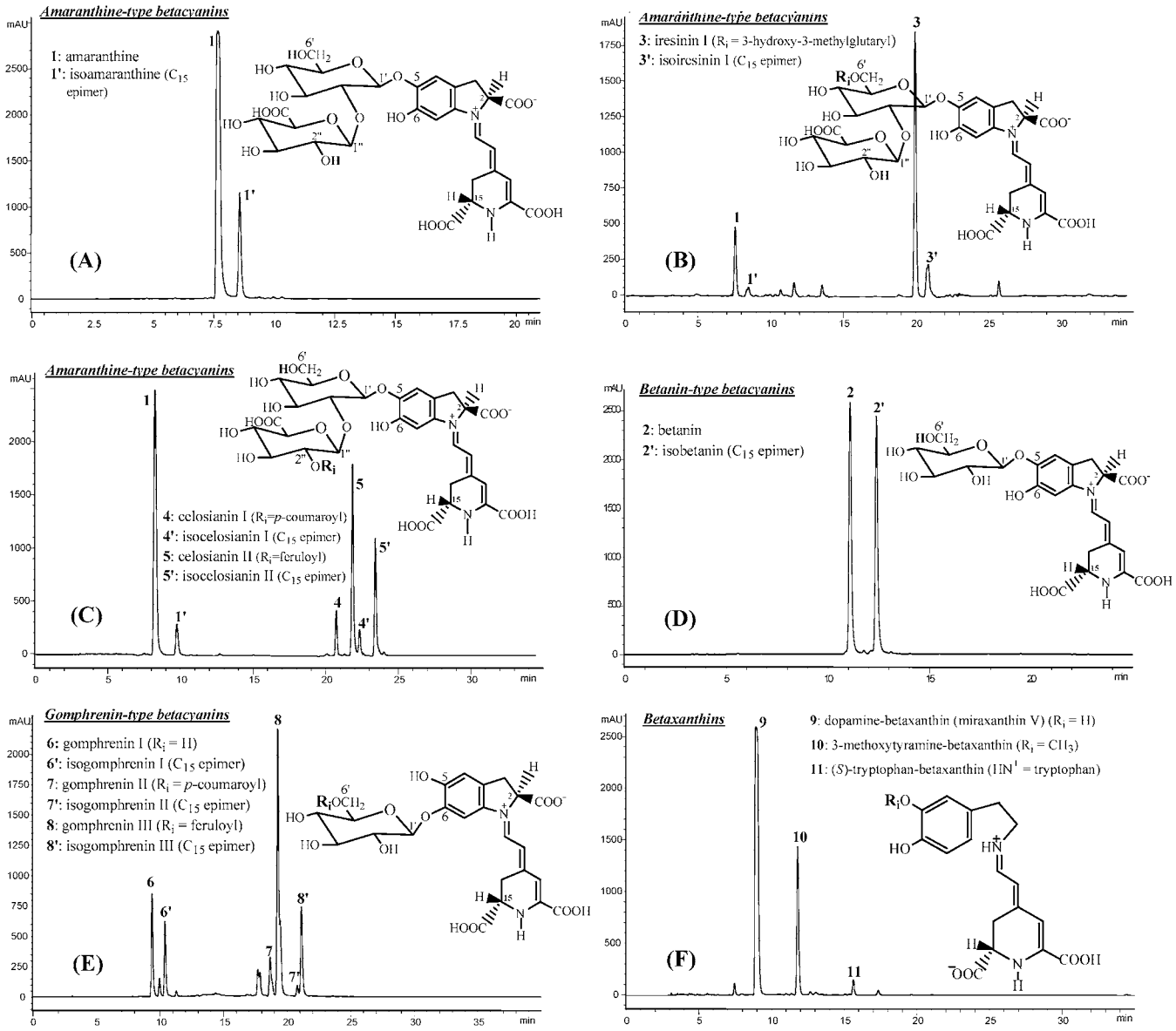

Figure 1. Structures and preparative HPLC elution profiles of betalains extracted from plants of the Amaranthaceae: (**A**–**C**) amaranthine type betacyanins from *A. tricolor*, *I. herbstii*, and *C. cristata* (violet); (**D**) betanin type betacyanins from red beet powder instead of *A. cruentus* (low betanin content); (**E**) gomphrenin type betacyanins from *G. globosa*; and (**F**) betaxanthins from *C. plumosa*. The isomeric forms (**1′**, **2′**, **3′**, **4′**, **5′**, **6′**, **7′**, and **8′**) are the C-15 epimers of corresponding betacyanins (not drawn). Peak numbers correspond to the numbers in **Table 1**.

(8.37/8.35 μM). To some extent, the results were similar to those of Kanner et al. (*15*) who reported that betanin and betaxanthins from beet roots had stronger antioxidant activity than catechin. The IC_{50} inhibition concentrations of linoleate peroxidation by betanin, betaxanthin (vulgaxanthin I), and catechin were 0.4, 1, and 1.2 μM. Additionally, inhibition (%) of DPPH$^\bullet$ radical at the same concentration of antioxidants is a direct parameter used to express antioxidant activity (*27*). The higher inhibition (%) represented higher scavenging activity of free radicals and stronger activity of antioxidants. For the same compounds, inhibition (%) and EC_{50} values gave similar trends in antioxidant activity (**Table 1**).

Another parameter of antioxidant activity is antiradical activity, defined as the initial slope calculated by linear regression in units of μmol of DPPH$^\bullet$/μmol of compound (*22*). The slopes (antiradical activity) in **Table 1** consistently matched results of antioxidant activity of the betalains and standard compounds tested as EC_{50} values. A higher absolute slope for antiradical activity indicates a higher concentration of DPPH$^\bullet$ needed to react with each compound, implying higher potential antioxidant activity. Additionally, the slope (antiradical activity) usually depends on the reaction kinetic behavior of different antioxidants at the same time and concentration. Different antioxidants had varied reaction rates for DPPH$^\bullet$ that produced different reaction kinetic behaviors (*23*, *26*). Reaction kinetic behavior was generally classified into three types. Ascorbic acid (**Figure 2A**) was the only example of rapid kinetic behavior in this study, reacting very rapidly with DPPH$^\bullet$ and reaching a steady state in less than 2 min. Most compounds tested were intermediate in behavior, reaching a steady state after about 90−120 min. Gomphrenin type betacyanins (**Figure 2B**) and three betaxanthins were rapid-intermediate, while betanin type and amaranthine type betacyanins were slow-intermediate. The third type was slow kinetic behavior and included rutin (**Figure 2C**), catechin, and ferulic acid, taking about 120−180 min to reach a steady state. Therefore, gomphrenin type betacyanins and three betaxanthins not only have higher antiradical activity but also

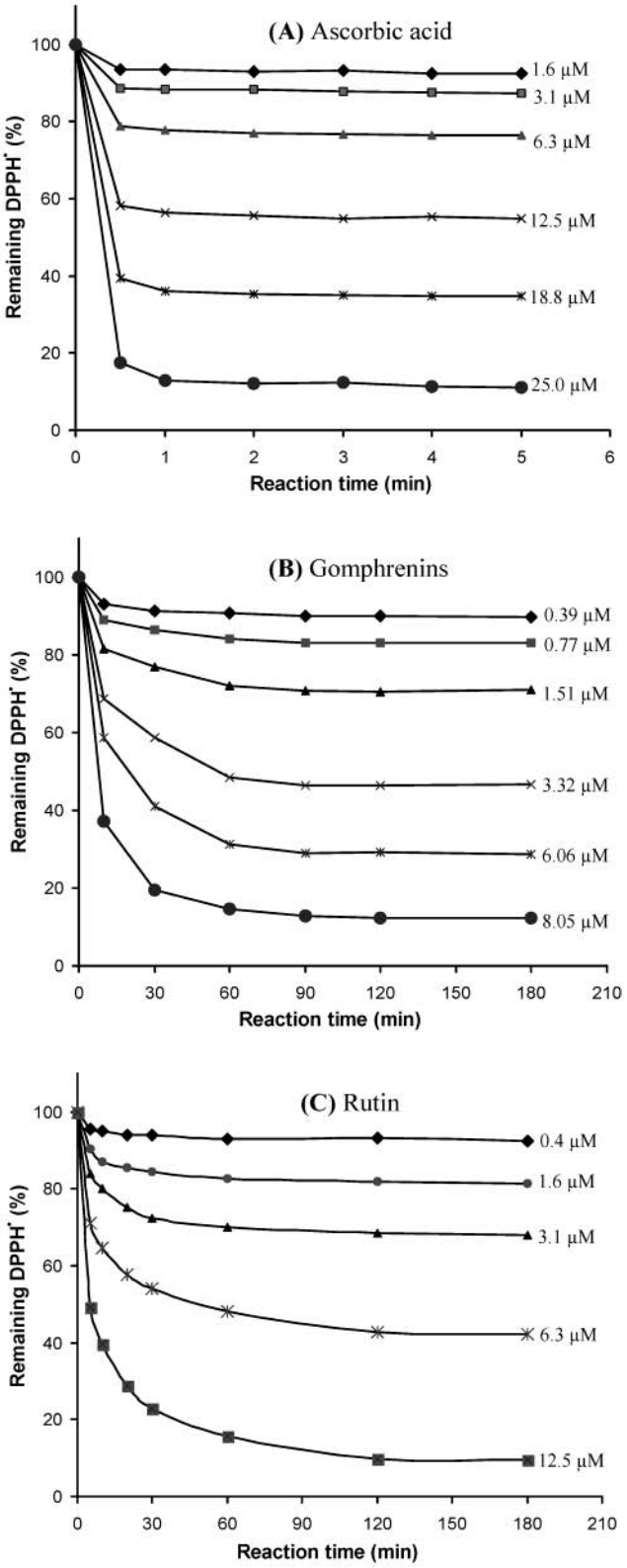

Figure 2. Three examples of reaction kinetics of the selected betalains and tested compounds (μM) at 60 μM DPPH•: (**A**) rapid behavior (ascorbic acid); (**B**) intermediate behavior (gomphrenin type betacyanins); and (**C**) slow behavior (rutin).

belong to rapid-intermediate kinetic behavior, indicating that they are highly efficient natural antioxidants.

Structure−Activity Relationship. According to the EC_{50} and inhibition (%) values in **Table 1**, antioxidant activity of the tested betalain compounds decreased in the following order: simple gomphrenins > acylated gomphrenins > dopamine-betaxanthin > (*S*)-tryptophan-betaxanthin > 3-methoxytyramine-betaxanthin > betanin/isobetanin > celosianins > iresinins > amaranthine/isoamaranthine. This sequence indicated that the DPPH• radical scavenging activity of the betalain compounds was due to their hydrogen-donating ability that depended mainly on their different structural features.

Although betalain compounds are not flavonoids, they possess a monoglucosylated *O*-diphenol group and a cyclic amine group, the partial structure of which resembles that of ethoxyquin, a very strong antioxidant (*15*). Betalain molecules were very good electron donors. Cuvelier et al. (*34*), Fukumoto and Mazza (*22*), and Son and Lewis (*27*) discussed the relationships between antioxidant activity and structures of phenolic acids, anthocyanins, and flavonoids. Generally, an increase in the number of hydroxyl groups (−OH) or other H-donating groups (═NH; −SH) in the molecular structure led to higher antioxidant activity. The position of hydroxyl groups and glycosylation of aglycones also affected activity. Our observations on the betalains in the present study were basically consistent with their findings concerning other compounds. From **Figure 1**, different betalain (betacyanin and betaxanthin) molecules contain various numbers of −OH and ═NH groups. Betacyanins are glycosides or acylglycosides of the aglycones (betanidin/isobetanidin), which are the basic structural units of nearly all betacyanins (**Figure 1A**−**E**). Betaxanthins are formed from betalamic acid and various amines or amino acids (**Figure 1F**). Among all betalains, the three yellow betaxanthins exhibited better antioxidant activity than most of the red-violet betacyanins, with the exception of gomphrenin type betacyanins (**Table 1**). A comparison of their structures shows that the betaxanthins studied contain 2−3 imino groups (═NH) and 1−2 hydroxyl groups (−OH) in the amines (dopamine or 3-methoxytyramine) or amino acid (tryptophan) moiety and betalamic acid moiety, while all betacyanins tested have only one −OH and one ═NH in the betanidin/isobetanidin moiety. The −OH groups on the sugar units of betacyanins are not H-donors and do not have any activity. Previous studies gave conflicting results on the antioxidant activity of the same betacyanins and betaxanthins from beet roots. Zakharova and Petrova (*13*) reported that yellow vulgaxanthins (glutamic acid or glutamine-betaxanthins) had higher antioxidant activity than red-violet betanin/isobetanin, but Escribano et al. (*12*) and Kanner et al. (*15*) showed opposite results. This was likely due to different purification procedures for betalain samples and various assessment methods for antioxidant activity applied in their studies. The final purification in those studies was not done by preparative HPLC. Zakharova and Petrova (*13*) determined inhibition (%) of lipid peroxidant (LPO), whereas Escribano et al. (*12*) used the $ABTS^{\bullet+}$ method. The concentrations were estimated using a molar absorptivity (ϵ) of 60 000 for betaxanthins in Kanner et al. (*15*). However, many reports gave ϵ = 40 000−50 000 for betaxanthins (*20*, *35*, *36*).

Among the three betaxanthins, dopamine-betaxanthin showed stronger activity than (*S*)-tryptophan-betaxanthin and 3-methoxytyramine-betaxanthin (**Table 1**). The dopamine structure containing two hydroxyl groups (−OH) and one imino group (═NH) can increase its H-donating ability and activity. Caffeic acid amide analogues (containing imino group) with the dopamine structure are very strong antioxidants (*27*). However, gomphrenin type betacyanins exhibit the strongest antioxidant activity not only among all tested betacyanins but also among all tested betalains, probably due to the position (steric hindrance) of hydroxyl groups. Gomphrenin type betacyanins

contain an −OH group at C-5 of betanidin/isobetanidin (**Figure 1E**), but betanin type and amaranthine type betacyanins contain −OH groups at C-6 (**Figure 1A−D**). We surmise that the presence of the −OH group at C-5 of betanidin/isobetanidin may increase O−H-bound dissociation energy and significantly improve H-donating ability and activity as compared to that at C-6. Therefore, gomphrenin type betacyanins possessed markedly higher antioxidant activity than betanin type and amaranthine type betacyanins. However, this assumption regarding the mechanism needs to be proven. An analysis of the structure−activity relationship between amaranthine type and betanin type betacyanins revealed that the former, with two sugar units (**Figure 1A−C**), exhibited lower antioxidant activity than the latter, with only one sugar unit (**Figure 1D**), because the higher extent of glycosylation of betanidin/isobetanidin led to lower activity. Zakharova and Petrova (*13*) reported similar findings that betanin from red beet had a higher inhibition (%) of lipid peroxidation than amaranthine from *A. tricolor*.

In addition, the structure−activity relationship comparison between acylated betacyanins and simple (nonacylated) betacyanins was not clear. The antioxidant activity of acylated amaranthines (celosianins and iresinines) was higher than or similar to that of amaranthine/isoamaranthine (**Table 1**). This probably resulted from acyl groups (i.e., feruloyl and *p*-coumaroyl) in celosianin molecules. The −OH on the acyl groups might be the H-donor because ferulic acid (EC_{50} = 19.35 μM in **Table 1**) and *p*-coumaroyl acid are weak antioxidants (*22*). Nevertheless, acylated gomphrenins with feruloyl and *p*-coumaroyl groups exhibited lower activity than simple gomphrenins. This was in contrast to the results for acylated amaranthines. This might result from the influence on H-donor by the interaction of acyl groups and glycosylation of aglycones. Further investigation of this mechanism is required.

In conclusion, our results clearly showed that betalains from plants of the Amaranthaceae, especially red-violet gomphrenin type betacyanins and yellow betaxanthins, demonstrated very strong antioxidant activity, as compared to typical antioxidants (ascorbic acid, rutin, and catechin), suggesting that the betalains may become a useful source of both natural antioxidants and natural colorants. This study also revealed that antioxidant activity of different betalains generally depended on their chemical structures. The free radical scavenging activity of the betalains increased with the number of hydroxyl groups and imino groups in the molecule. The C-5 position of the hydroxyl group on aglycones in the betalain molecules significantly improved activity, and more glycosylation of aglycones clearly reduced activity.

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