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ARTICLE

In Vitro Synthesis of Betaxanthins Using Recombinant DOPA 4,5-Dioxygenase and Evaluation of Their Radical-Scavenging Activities

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Betalamic acid, the chromophore of betaxanthins, was enzymatically synthesized on a large scale from L-dihydroxyphenylalanine (L-DOPA) using recombinant *Mirabilis jalapa* DOPA 4,5-dioxygenase. After synthesis, proline was directly added to the concentrated reaction mixture to generate proline-betaxanthin. The molecular mass and nuclear magnetic resonance spectrum of the purified product were identical to those previously reported for proline-betaxanthin. Twenty-four betaxanthin species were synthesized by the condensation reaction of purified betalamic acid and amino acids or amines. An HPLC protocol was established for identifying the different betaxanthin species. Proline-, dopamine-, and γ-aminobutyric acid (GABA)-betaxanthins were prepared as representative betaxanthins under large-scale conditions, and their 1,1-diphenyl-2-picrylhydrazyl radical-scavenging activities were compared against those of known antioxidants. GABA-betaxanthin showed comparatively low activity, whereas dopamine-betaxanthin had similar activity to the red pigment betanin and the anthocyanin cyanidin 3-glucoside. Proline-betaxanthin had the highest activity of the three synthesized compounds and was similar to the flavonoid quercetin.

KEYWORDS: Antioxidant activity; betalamic acid; betaxanthin; DOPA 4,5-dioxygenase; *Mirabilis jalapa*

INTRODUCTION

Betalains are water-soluble nitrogenous pigments that are often used as colorant additives in foods. Crop plants such as red beet (*Beta vulgaris* L.), Swiss chard (*Beta vulgaris* L. ssp. *cicla* [L.]), ulluco (*Ullucus tuberosus*), and cactus fruits (*Opuntia ficus-indica*) are known to contain betalain pigments (*1*−*5*). The pigments are present in plant species of the order Caryophyllales with the exception of the Caryophyllaceae and Molluginaceae families in which anthocyanins do not occur (*6*). Betalains are composed of two groups, red betacyanins and yellow betaxanthins (for reviews, see refs *7* and *8*). All betalains contain betalamic acid as the chromophore. Betalamic acid conjugated with *cyclo*-DOPA 5-*O*-glucoside forms betacyanins, which appear red. The yellow pigment betaxanthin is synthesized in vivo by the conjugation of betalamic acid with an amino acid or amine. Because some betalain molecules have a polyphenol-like structure, betalains are expected to show antioxidant activity similar to anthocyanins. Betanin purified from red beet extracts does indeed display strong 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity (*9*). In the case of betaxanthins, analysis of 3-methoxytyramine-betaxanthin and dopamine-betaxanthin extracted and purified from *Celosia plumosa* and of dopamine-betaxanthin semisynthesized using alkaline hydrolysis of betalain showed that they have DPPH radical-scavenging activities (*7*, *10*). Although there are claims that betalains might have antioxidant activity, most of the studies reporting this property did not use purified betalain molecules but rather used plant extracts that contained several betalain compounds and also other contaminating substances. With a few exceptions, the antioxidant activity of each type of betalain molecule has not yet been precisely evaluated and, furthermore, the possibility of antioxidant activity due to residual contaminants in the extracts remains (*2*, *5*, *11*−*13*). The reason for the paucity of reports on the antioxidant activity of pure betalains, especially betaxanthins, is the difficulty of isolating and purifying sufficient amounts of betaxanthins from plant extracts. It is possible to prepare betalamic acid by alkaline hydrolysis of betacyanin or betaxanthin, and the betalamic acid can then be condensed with an amino acid or amine to yield the desired betaxanthin molecules (*14*). However, the methods are too laborious to prepare sufficient amounts or many kinds of betaxanthin molecules.

In plants, betalamic acid is synthesized by DOPA 4,5-dioxygenase (DOD), which catalyzes L-DOPA to 4,5-*seco*-DOPA followed by spontaneous chemical recyclization to form betalamic acid. In a previous study, the *DOD* gene of *Portulaca grandiflora* and of *B. vulgaris* was identified (*15*). On the basis of these plant *DOD* cDNA sequences, we succeeded in isolating a cDNA encoding DOD from *Mirabilis jalapa* (MjDOD). A recombinant MjDOD protein was expressed in *Escherichia coli* and showed DOD activity in an in vitro reaction to synthesize betalamic acid (*16*). Here, we used recombinant MjDOD for large-scale synthesis of betalamic acid and efficiently purified the betalamic acid using medium-pressure flash liquid chromatography

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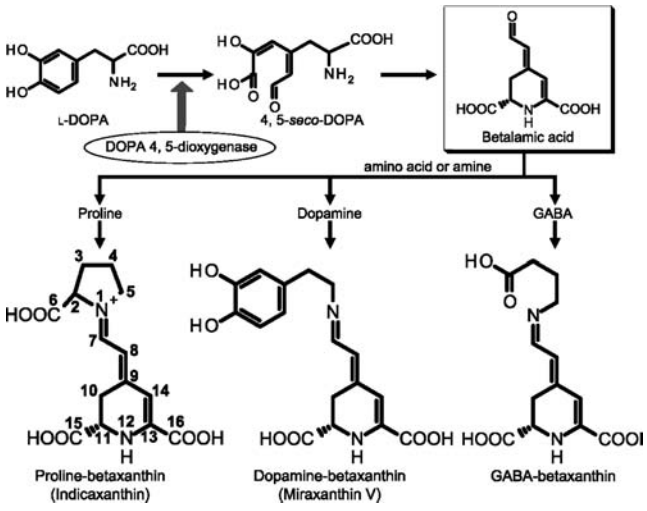

Figure 1. Scheme for betaxanthin synthesis using the DOPA 4, 5-dioxygenase enzymatic reaction. L-DOPA was catalyzed by DOPA 4,5-dioxygenase (DOD) to produce 4,5-*seco*-DOPA. It is then possible to generate betalamic acid chemically by spontaneous recyclization. Betalamic acid conjugated with amino acid or amine yields betaxanthins that are yellow.

(FLC) (**Figure 1**). Enzymatically synthesized betalamic acid was supplied to a condensation reaction with amino acids or amines to obtain 25 different betaxanthin molecular species. From these betaxanthins, we selected proline-, dopamine-, and GABA-betaxanthins for large-scale synthesis followed by purification by FLC and evaluated their respective radical-scavenging activities for DPPH.

MATERIALS AND METHODS

Chemicals. Dopamine HCl, L-dihydroxyphenylalanine (L-DOPA), and tyramine were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). L(+)-Ascorbic acid, L-alanine, L(+)-arginine hydrochloride, L-asparagine monohydrate, L-aspartic acid, L-cysteine hydrochloride, γ-amino-*n*-butyric acid (GABA), L(+)-glutamine, L-glutamic acid, glycine, L(+)-isoleucine, L-histidine, L-hydroxyproline, L-leucine, L(+)-lysine hydrochloride, L-methionine, L-phenylalanine, L(−)-proline, L-serine, L(−)-threonine, L-tryptophan, L-tyrosine, and L-valine were purchased from Wako Pure Chemicals Ltd. (Osaka, Japan). Quercetin hydrate and cyanidin 3-*O*-β-D-glucoside (Cy3G) were purchased from Sigma-Aldrich (St. Louis, MO) and Funakoshi Corp. (Tokyo, Japan), respectively. Betanin was purified from red beet extract purchased from Tokyo Kasei Kogyo. Red beet extract was dissolved in 0.1% acetic acid and then applied to an HP-20 resin (Mitsubishi Chemical Corp., Tokyo, Japan) column. After the column had been washed with 0.1% aqueous acetic acid, the pigments were eluted with methanol. The eluent was then separated using a medium-pressure liquid chromatograph (FLC, Yamazen Flash Liquid Chromatography YFLC-AI-580, Yamazen Corp., Osaka, Japan) equipped with reverse-phase columns (Hi-Flash columns, ODS-SM: 50 μm, i.d. 20 × 65 mm column, followed by i.d. 26 × 100 mm column, Yamazen) using a linear gradient elution (20 mL min^{-1}) of 15−50% methanol in solvent A (0.1% aqueous acetic acid) for 20 min. A second round of purification using the same column setup was performed with a linear gradient elution (20 mL min^{-1}) of 15−40% methanol in solvent A for 20 min.

Enzymatic Synthesis of Betalamic Acid Using DOPA 4,5-Dioxygenase from *M. jalapa*. *M. jalapa DOPA 4,5-dioxygenase* (*MjDOD*) cDNA in pDEST17 (Invitrogen, Carlsbad, CA) was transformed into *E. coli* BL21-AI (Invitrogen), an arabinose-inducible strain. The transformants were grown overnight at 30 °C in 5 mL of Luria−Bertani (LB) medium containing 50 μg mL^{-1} ampicillin. Four milliliters of precultured cells were transferred into 800 mL of LB medium containing 50 μg mL^{-1} ampicillin. The cells were inoculated and cultured until OD_{600} reached 0.5−0.6 at 30 °C; 8 mL of 20% arabinose was then added, and the cells were cultured for a further 3 h at 16 °C. The cells were harvested by centrifugation at 1700*g* for 15 min and were washed with 80 mL of the extraction buffer (100 mM potassium phosphate, pH 7.2) by centrifugation, and resuspended in 60 mL of the extraction buffer. The cells were disrupted by sonication using an ultrasonic disintegrator (UD-201, Tomy Seiko Co. Ltd., Tokyo, Japan). After centrifugation of the lysate at 20000*g* for 20 min, the supernatant was dialyzed with 2 L of buffer (100 mM potassium phosphate, pH 7.2, containing 50 μM $FeSO_4$) at 4 °C and used as the crude extract for the enzyme reaction. A reaction mixture (800 mL) of 1 mM L-DOPA, 10 mM ascorbic acid, 50 μM $FeSO_4$, 100 mM potassium phosphate, pH 7.2, and 20−40 mL of the crude extract was prepared. The reaction mixture was incubated at 30 °C for 3 h followed by the addition of 800 mL of 100% methanol to terminate the enzyme reaction. After filtration through filter paper to remove insoluble debris, the reaction mixture was evaporated to 30 mL and used directly for large-scale synthesis of proline-, dopamine-, and GABA-betaxanthins. For the small-scale synthesis of 21 amino acid- and 4 amine-betaxanthins, the betalamic acid was further purified using an FLC equipped with two columns directly connected in series of Toyopearl SuperQ-650 M (i.d. 20 × 65 mm column followed by i.d. 26 × 100 mm column; Tosoh Co. Ltd., Tokyo, Japan) using water and solvent B (2 M NaCl) and a stepwise elution program at 20 mL min^{-1}: 100% water for 0−2 min; 0−24% B in water for 2−6 min; and, 24% B in water for 6−10 min. The yellow fractions were combined and further purified using two Hi-Flash ODS-SM columns directly connected in series (i.d. 20 × 65 mm column followed by i.d. 26 × 100 mm column; Yamazen) by solvent A and methanol following a stepwise elution program at 20 mL min^{-1}: 100% A for 0−5 min; 0−35% methanol in A for 5−20 min; and 35% methanol in A for 25−30 min. The yellow fractions were combined and loaded on two ODS-WAKOSIL 25-C-18 columns directly connected in series (i.d. 20 × 65 mm column followed by i.d. 26 × 100 mm column; Wako Pure Chemicals) to be purified by solvent C (0.1% aqueous trifluoroacetic acid (TFA)) and methanol following a stepwise elution program at 20 mL min^{-1}: 100% C for 0−5 min; 5% methanol in C for 5−10 min; 10% methanol in C for 10−15 min; 15% methanol in C for 15−20 min; and 20% methanol in C for 20−25 min. After purification, the quality of the betalamic acid was analyzed by HPLC-DAD using the LaChromElite System (pump L-2130, UV−vis detector L-2420, Hitachi High-Technologies, Tokyo, Japan) equipped with a reversed-phase column of Handy ODS (i.d. 4.6 mm × 250 mm; Wako Pure Chemicals) separated by linear gradient elution (1 mL min^{-1}) from 7% methanol to 65% in solvent D (1.5% aqueous phosphoric acid) for 15 min.

Synthesis and Purification of Betaxanthins. For large-scale synthesis of proline-, GABA-, and dopamine-betaxanthin, three independently synthesized batches of betalamic acid in 50% methanol were each evaporated to approximately 30 mL, and powdered proline, dopamine, or GABA was added directly into each batch to form a saturated solution; these solutions were incubated overnight at 4 °C. The reaction mixtures were allowed to evaporate completely, and each product was dissolved in 0.1% TFA. Purification of betaxanthins was performed by FLC equipped with two ODS-WAKOSIL 25-C-18 columns directly connected in series (i.d. 20 × 65 mm column followed by i.d. 26 × 100 mm column; Wako Pure Chemicals) by stepwise elution (20 mL min^{-1}). At the first step purification of all proline-, dopamine-, and GABA-betaxanthins, the products were purified by the following stepwise elution: 0−5 min, 100% C; 5−10 min, 5% methanol in C; 10−15 min, 10% methanol in C; 15−20 min, 15% methanol in C; 20−25 min, 20% methanol in C; 25−30 min, 25% methanol in C; 30−35 min, 30% methanol in C; and, 35−40 min, 35% methanol in C. A second round of purification using the same column was performed using the stepwise elution program 0−5 min, 100% C; 5−25 min, 0−30% for proline-betaxanthin or 0−35% for dopamine-betaxanthin or 0−25% for GABA-betaxanthin methanol in C; 25−30 min, 30 or 35 or 25% methanol in C corresponding to the final step concentration of each betaxanthin.

The molecular mass of proline-betaxanthin was confirmed by electrospray ionization−mass spectrometry (ESI-MS) (AccuTOF MS, JMS-T100LC, JEOL, Tokyo, Japan). Nuclear magnetic resonance (NMR) spectra (^{1}H NMR, ^{13}C NMR, ^{1}H−^{1}H COSY, HMBC, and HMQC) were recorded on a JEOL JNM-ECA500 spectrometer. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR chemical shifts were referenced to the residual solvent (CD_3OD) signal at δ_H 3.30 and δ_C 49.8, respectively.

For the small-scale condensation reaction of betalamic acid with the 21 amino acids and 4 amines, a 1 mL solution of betalamic acid in water was prepared and powdered individual amino acids and amines were directly added and dissolved at the individual saturated concentrations for L-alanine, L(+)-arginine, L-asparagine, L-aspartic acid, L-cysteine, dopamine, L-DOPA, GABA, L-glutamic acid, L(+)-glutamine, glycine, L-histidine, L-hydroxyproline, L(+)-isoleucine, L-leucine, L(+)-lysine, L-methionine, L-phenylalanine, L(−)-proline, L-serine, L(−)-threonine, L-tryptophan, tyramine, L-tyrosine, L-valine. The condensation reactions were performed overnight at 4 °C. Each sample was filtered through an Ultra Free centrifugal filter (Millipore Corp., Bedford, MA) and analyzed by HPLC-DAD using the same method as described for separating betalamic acid. Synthesized betaxanthins were separated using an HPLC-DAD system equipped with a reversed-phase column of Handy ODS (i.d. 4.6 mm × 250 mm) with linear gradient elution (1 mL min^{-1}) from 7 to 65% methanol in 0.1% aqueous formic acid for 15 min. The separated betaxanthin fractions were retrieved and then subjected to ESI-MS analysis. The mixture of semisynthesized betaxanthin standards was separated by HPLC using a linear gradient elution (1 mL min^{-1}) from 5 to 8% methanol in solvent D for 5 min, from 8 to 13% for 5 min, from 13 to 21% for 5 min, from 21 to 30% for 5 min, from 30 to 38% for 5 min, and from 38 to 43% for 5 min.

DPPH-HPLC Analyses. The protocol for evaluation of DPPH radical-scavenging activity was based on previous studies and a modified HPLC method (*17*, *18*). Each reaction mixture (1 mL) contained 250 nmol of DPPH and 5 nmol of proline-betaxanthin, dopamine-betaxanthin, GABA-betaxanthin, Cy3G, betanin, quercetin, or ascorbic acid. The DPPH radical-scavenging reaction was performed in the dark for 20 min at room temperature. After the reaction, each sample was analyzed by HPLC-DAD using a LaChromElite System (pump L-2130, UV−vis detector L-2420) equipped with a COSMOSIL ODS column (i.d. 4.6 × 50 mm) and separated by linear gradient elution (1 mL min^{-1}) from 63% acetonitrile to 65% in water for 10 min. The decrease in absorbance of DPPH at 520 nm was monitored by the detector, and DPPH radical-scavenging activity was expressed using the parameter of RS% by the following equation: inhibition of DPPH absorbance = $100 \times (A_{control} - A_{sample})/A_{control}$. Each sample was measured in three independent reactions, and the data are shown as the mean value and standard error.

RESULTS AND DISCUSSION

Large-Scale Synthesis of Betalamic Acid Using Recombinant MjDOD Protein and Establishment of a Purification Method by FLC. In our previous study, we succeeded in detecting DOD activity in a preparation from *E. coli* transformed with a cDNA encoding DOD of *M. jalapa* in an expression vector (*16*). Using this expression system, we established a method for large-scale production of betalamic acid combined with purification using medium-pressure FLC. Expression of MjDOD protein in *E. coli* was efficiently induced by the addition of arabinose at a final concentration of 0.2% and culturing for 3 h at 16 °C. It was found that the crude extract prepared from transformed *E. coli* just after dialysis could be directly used for the large-scale synthesis without degradation of the synthetic product, betalamic acid. Recent advances in techniques for purification of plant secondary metabolites involve the use of a combination of open column chromatography and HPLC, particularly with ODS-based resin. However, ODS-based resins were unable to retain a large amount of betalamic acid. Therefore, we did not use the ODS column for the first-step purification but instead used FLC equipped with an anion exchanger resin packed column that retained betalamic acid with high efficiency. First-step purification was accomplished by 10 min of FLC operation. Partially purified betalamic acid fractions were further purified using FLC equipped with ODS resin packed columns, which retained a small amount of betalamic acid but nevertheless worked well for the second and third purification steps (**Figure 2A**). After purification, an attempt was made to obtain the structure of betalamic acid by NMR, but this failed to yield good spectral data because of the instability of

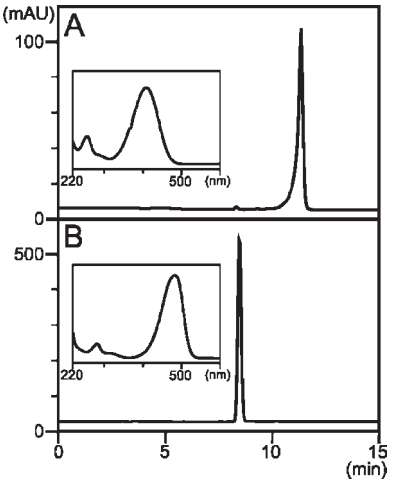

Figure 2. HPLC elution profiles of purified betalamic acid (monitored at 405 nm) and proline-betaxanthin (monitored at 470 nm): (**A**) betalamic acid; (**B**) proline-betaxanthin. Insets in **A** and **B** show the diode array spectrum of each pigment obtained by HPLC-DAD.

betalamic acid. To circumvent the instability of the betalamic acid, powdered proline was directly added to a freshly prepared betalamic acid solution, which was then incubated overnight at 4 °C. Conjugation of betalamic acid and proline produces proline-betaxanthin, which has a notably higher stability than betalamic acid, thus making it suitable for purification using FLC and for spectral analysis. From an 800 mL reaction mixture containing 1 mM L-DOPA (157.75 mg), we obtained a yield of 25.51 mg of proline-betaxanthin (mol percent yield = 10.3%); the final purification HPLC profiles are shown in **Figure 2B**. A molecular mass of 309 was obtained for the product at $[M + H]^+$ by ESI-MS. The ^1H, ^{13}C, $^1H-^1H$ COSY, HMBC, and HMQC NMR spectra showed the same chemical shift as has been previously reported for betaxanthins (**Table 1**) (*19*). Our analyses therefore indicate that synthesis of betalamic acid and betaxanthin using a recombinant protein was successful.

Properties of Betaxanthins and Betaxanthin Standards. Synthesized and purified betalamic acid was conjugated with L-alanine, L(+)-arginine, L-asparagine, L-aspartic acid, L-cysteine, dopamine, L-DOPA, GABA, L-glutamic acid, L(+)-glutamine, glycine, L-histidine, L-hydroxyproline, L(+)-isoleucine, L-leucine, L(+)-lysine, L-methionine, L-phenylalanine, L(−)-proline, L-serine, L(−)-threonine, L-tryptophan, tyramine, L-tyrosine, or L-valine to synthesize 25 betaxanthin species individually at a small scale (**Table 2** and **Figure 1**). Twenty-four of the synthesized betaxanthins (the exception was cysteine-betaxanthin) were used to establish an HPLC analytical method that permitted identification of each betaxanthin species (**Figure 3A**). Furthermore, a mixture of 24 kinds of betaxanthin species was separated using the HPLC analytical methods with similar resolution (**Figure 3B**). Twenty betaxanthin species showed different retention times under optimal analytical conditions; the exceptions were the pairs hydroxyproline-betaxanthin and aspartic acid-betaxanthin and tyramine-betaxanthin and valine-betaxanthin, with each pair showing similar retention times (**Table 2** and **Figure 3**). The retention times of the betaxanthins differed from those previously reported; this was the result of the use of HPLC analytical methods different from those of earlier reports. Nevertheless, the wavelengths of maximum absorbance and the molecular masses obtained by MS were essentially identical to those in the literature (*2*−*4*).

The different reaction mixtures varied in color; this was probably caused by differences in the wavelength of maximum

Table 1. ^{1}H and ^{13}C NMR Data of Each Proline-Betaxanthin[a]

atom	reference δ(H)	semisynthetics δ(H)	reference δ(C)	semisynthetics δ(C)
H−C(2)	4.63 (dd, J = 3.5, 8.4)	4.62 (dd, J = 2.9, 9.7)	67.6	68.4
Ha−C(3)	2.32	2.35	29.6	29.9
Hb−C(3)	2.19	2.18		
Ha−C(4)	2.03−2.10 (m)	2.03 (m)	23.1	24.5
Hb−C(4)	2.00	2.03 (m)		
Ha−C(5)	3.62−3.71	3.60−3.69	49.8	50.4
Hb−C(5)	3.62−3.71	3.60−3.69		
C(6)			175.6	175.6
H−C(7)	8.19 (d, J = 12.2)	8.29 (d, J = 12.1)	156.6	159.3
H−C(8)	6.02 (d, J = 12.2)	6.02 (d, J = 12.1)	107.7	110.7
H−C(9)			163.7	164.3
Ha−C(10)	3.28	3.27	26.4	27.9
Hb−C(10)	3.04	3.00		
H−C(11)	4.43 (t, J = 6.5)	4.43 (t, J = 6.3)	52.5	51.8
H−C(13)			153.0	150.4
H−C(14)	6.15 (s)	6.10 (s)	103.6	110.7
H−C(15)			175.5	175.5
H−C(16)			166.6	166.1

[a] Solvent data: D_2O containing 0.1% *d*-TFA; T = 298. Reference: proline-betaxanthin isolated from Swiss chard (*19*). Semisynthetics: proline-betaxanthin semisynthesized using recombinant protein MjDOD.

Table 2. Retention Times, Maximum Absorbance Wavelengths, and Molecular Masses of Semisynthesized Betaxanthin Standards

	betaxanthin	t_R (min)	λ_{max} (nm)	$[M + H]^+$ (m/z)
1	histidine-betaxanthin	4.640	473	349
2	lysine-betaxanthin	5.333	467	340
		8.720	454	340
3	asparagine-betaxanthin	6.267	470	326
4	arginine-betaxanthin	6.693	469	368
5	serine-betaxanthin	7.333	468	299
6	glutamine-betaxanthin	8.293	470	340
7	aspartic acid-betaxanthin	8.897	470	327
8	hydroxyproline-betaxanthin	8.960	481	325
9	glycine-betaxanthin	9.280	466	269
10	threonine-betaxanthin	10.773	469	313
11	glutamic acid-betaxanthin	12.320	470	341
12	alanine-betaxanthin	14.267	466	283
13	GABA-betaxanthin	15.867	457	297
14	proline-betaxanthin	16.133	478	309
15	DOPA-betaxanthin	19.093	472	391
16	dopamine-betaxanthin	20.587	459	347
17	tyrosine-betaxanthin	21.840	472	375
18	methionine-betaxanthin	22.587	471	343
19	tyramine-betaxanthin	22.933	459	331
20	valine-betaxanthin	23.067	470	311
21	isoleucine-betaxanthin	27.760	471	325
22	leucine-betaxanthin	28.213	471	326
23	phenylalanine-betaxanthin	28.907	473	359
24	tryptophan-betaxanthin	(i) NI[a]	513	NI
		(ii) 29.173	473	398

[a] (i) and (ii) correspond to the peaks of the HPLC chromatogram in **Figure 4D**. NI, we were not able to identify the molecular mass by MS.

absorbance, which determines the color shown by each betaxanthin (**Table 2**). In addition to differences in the wavelength of maximum absorbance, the betaxanthin product concentration in each mixture might also influence the color of each betaxanthin. The concentration of betaxanthin product in each mixture can vary because the efficiency of the condensation reaction can differ depending on the amino acid or amine present. Some betaxanthins show characteristic colors as a consequence of their different wavelengths of maximum absorbance (data not shown). Representative betaxanthins, such as glutamine-betaxanthin (**Figure 4A**) and serine-betaxanthin (**Figure 4B**), showed the typical yellow of betaxanthins composed of a single peak. In contrast, tyramine-betaxanthin was brownish in solution. This probably resulted from a high concentration of the product, because tyramine-betaxanthin has a wavelength of maximum absorbance (470 nm) similar to that of other betaxanthins. Alternatively, the brownish color may have been due to degradation of tyramine-betaxanthin molecules. The compound assumed to be cysteine-betaxanthin showed the expected yellow appearance after synthesis; however, the color disappeared during storage at room temperature because of degradation. Lysine-betaxanthin and tryptophan-betaxanthin were orange and scarlet, respectively. The HPLC-DAD analysis of lysine-betaxanthin produced two peaks (**Figure 4C**), whereas tryptophan-betaxanthin had two major and several minor peaks in the reaction mixture (**Figure 4D**). The colors of these betaxanthins were probably derived from a combination of multiple betaxanthin products in the reaction mixture. Production of a number of betaxanthins might result from the presence of multiple nucleophilic-like amino groups in the structures of lysine and tryptophan. Because one or more of these nucleophilic groups might interact with the carbonyl group of betalamic acid, multiple betaxanthin products might be synthesized. We were unable to identify which peaks of the in vitro synthesized lysine- and tryptophan-betaxanthins corresponded to those produced by betaxanthins of yellow Swiss chard, cactus pear, or yellow beet in vivo. It is known that the amine of the amide bond in glutamine and asparagine has low reactivity because of its intramolecular electron conjugation. A single betaxanthin synthesized with glutamine and asparagine might be dominantly synthesized by conjugation not with a nitrogen atom of the amide group but with another nitrogen atom.

Antioxidant Activity of Betaxanthins. It has been reported that betacyanins and betaxanthins show antioxidant activity (*3*−*5*, *9*, *11*−*13*, *19*−*22*). Proline-betaxanthin, GABA-betaxanthin, and dopamine-betaxanthin are present in yellow Swiss chard, cactus pear, and yellow beet (*2*−*5*). We prepared GABA-betaxanthin and dopamine-betaxanthin by a large-scale condensation reaction of betalamic acid followed by purification using FLC and assessed the antioxidant activities of these synthetic compounds, together with that of proline-betaxanthin. From the 800 mL reaction mixture in 1 mM L-DOPA (157.75 mg), we obtained yields of 15.79 mg of GABA-betaxanthin (mol percent yield = 6.6%) and

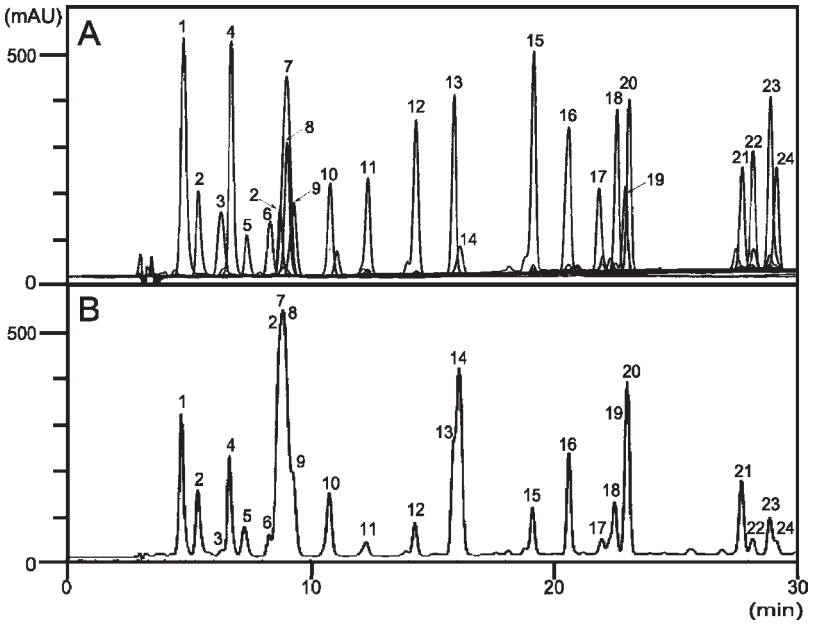

Figure 3. (**A**) HPLC chromatogram of the 24 betaxanthins separated individually and monitored at 470 nm. (**B**) HPLC separation profile of a mixture of 24 betaxanthins. The betaxanthin numbers correspond to those in **Table 2**.

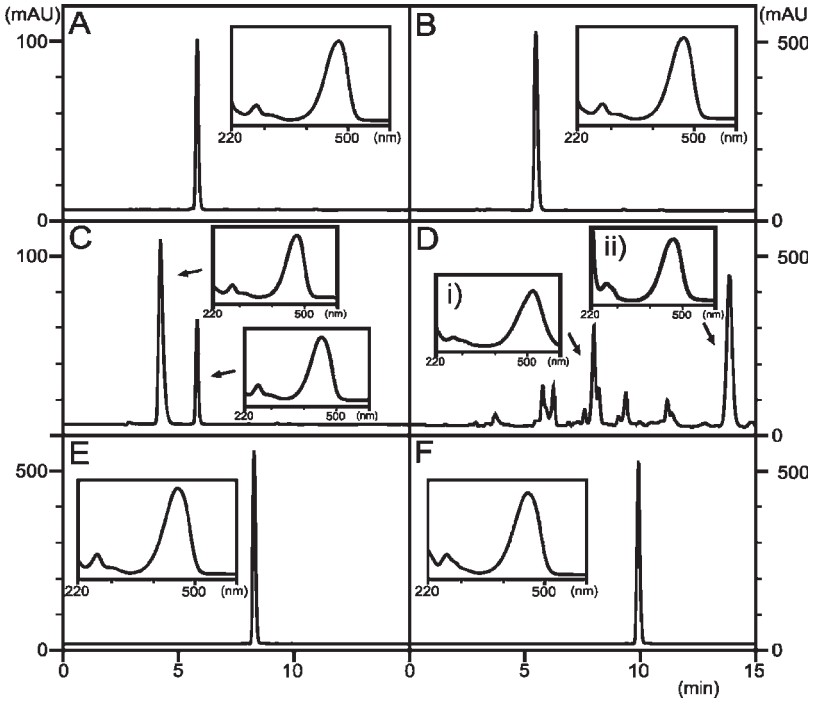

Figure 4. HPLC-DAD analysis of characteristic betaxanthins (monitored at 470 nm): (**A**) glutamine-betaxanthin; (**B**) serine-betaxanthin; (**C**) lysine-betaxanthin; (**D**) tryptophan-betaxanthin; (**E**) GABA-betaxanthin; (**F**) dopamine-betaxanthin. Insets in **A**−**F** show the absorption spectrum of each pigment obtained by HPLC analysis.

26.5 mg of dopamine-betaxanthin (mol percent yield = 9.5%) and the final purification HPLC profiles shown in **Figure 4E**,**F**. The DPPH radical-scavenging activities of proline-betaxanthin, GABA-betaxanthin, and dopamine-betaxanthin were evaluated using the DPPH-HPLC method (*23*, *24*). We also used DPPH-HPLC to evaluate betanin, the red pigment of betacyanin, Cy3G, a typical anthocyanin, quercetin, which has an antioxidant activity comparable to that of rutin, and ascorbic acid, an archetypal antioxidant. To decide the appropriate concentrations of the test substances for DPPH-HPLC, we evaluated the scavenging of DPPH radicals with six different concentrations of quercetin. We found that 5 μM (final concentration) quercetin showed a greater time-dependent linearity for decreasing absorbance than the other tested concentrations. Consequently, DPPH radical-scavenging activities were evaluated for all substances at a 5 μM final concentration. Although all of the betalain pigments showed DPPH radical-scavenging activity, their respective activities varied (**Figure 5**). GABA-betaxanthin showed the lowest activity of the compounds tested, whereas dopamine-betaxanthin showed scavenging activity similar to that of betanin and Cy3G. Proline-betaxanthin had the highest activity of the betaxanthins and was similar to that of quercetin.

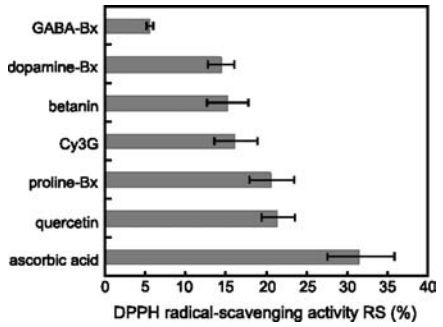

Figure 5. DPPH radical-scavenging activity of 5 μM samples at 250 μM DPPH. RS%, inhibition of DPPH absorbance = 100 × ($A_{control}$ − A_{sample})/$A_{control}$. GABA-Bx, γ-aminobutyric acid-betaxanthin; dopamine-Bx; dopamine-betaxanthin; Cy3G, cyanidin 3-glucoside; proline-Bx, proline-betaxanthin.

In this paper, we have established a system in which betalamic acid was synthesized in vitro on a large scale using a recombinant DOD protein; the betalamic acid was then used for preparation of betaxanthin species with amino acids and amines. This system has the considerable advantage of circumventing the laborious methods currently available for preparing small amounts of betaxanthins from large amounts of plant materials. We believe that the establishment of a method for preparing pure betaxanthins will greatly aid the assessment of their antioxidant activity, their radical-scavenging activity, and their possible benefits to human health.

ABBREVIATIONS USED

L-DOPA, L-dihydroxyphenylalanine; GABA, γ-aminobutyric acid; DPPH, 1,1-diphenyl-2-picrylhydrazyl; DOD, DOPA 4, 5-dioxygenase; FLC, flash liquid chromatography; Cy3G, cyanidin 3-glucoside.

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