Enzymatic Conversion of Precarthamin to Carthamin by a Purified Enzyme from the Yellow Petals of Safflower

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Precarthamin, a yellow precursor of carthamin, was efficiently isolated from the yellow petals of safflower (*Carthamus tinctorius* L.) with Sephadex LH-20 column chromatography and preparative HPLC, and identified with UV−vis and NMR spectrometry. The UV−vis spectrum of precarthamin showed λ_{max} of 238 and 406 nm in MeOH. The molar extinction coefficients of precarthamin at 406 nm in MeOH and 50 mM citrate buffer (pH 5.0) were 59 000 M^{-1} cm^{-1} and 45 400 M^{-1} cm^{-1}, respectively. The isolated and structurally identified precarthamin was converted to a red pigment by a homogeneously purified enzyme from the immature petals of safflower in 50 mM citrate buffer (pH 5.0). The enzymatically converted red pigment was identified as carthamin, a red pigment of safflower by TLC, HPLC, and UV−vis spectral analysis.

Keywords: *Safflower; Carthamus tinctorius; precarthamin; carthamin*

INTRODUCTION

Safflower (*Carthamus tinctorius* L.) is an annual herb of the compositae and has long been used as food colorants, dyes, and medicine in oriental countries. The flowers of safflower are very useful source of natural yellow and red colorants. However, the low content of red pigment, carthamin, is limiting factor to use of safflower for industrial purpose. As a part of works to improve the content of red pigment in the flowers of safflower, we studied enzymes involved in biosynthesis of carthamin and enzymatic synthesis of carthamin.

The petals of safflower contain yellow and red quinochalcone pigments. Yellow pigments are safflower yellow A (Takahashi et al., 1982), safflower yellow B (Takahashi et al., 1984), safflomin A (Onodera et al., 1981), safflomin C (Onodera et al., 1989), and precarthamin (Kumazawa et al., 1994; Kazuma et al., 1995). A red pigment of safflower, carthamin, is composed of two chalcone moieties which are fully conjugated (Obara and Onodera, 1979; Takahahi et al., 1982; Kim et al., 1996). Therefore, carthamin absorbs longer wavelength of light than those of other chalcone pigments and shows a red color.

The flower color of safflower is yellow just after flowering and gradually changes to red. The color transition of safflower is due to the conversion of yellow pigments to a red pigment, carthamin. A putative precursor of carthamin was isolated from the petals of safflower and converted to carthamin by an enzyme extract and a partially purified enzymes obtained from vegetative tissues and seedlings of safflower (Saito et al., 1983a,b, 1985). However, the structural determination of a putative precursor of carthamin, precarthamin, was carried out 10 years later (Kumazawa et al., 1994; Kazuma et al., 1995). Therefore, enzymatic conversion of a precursor, precarthamin with high purity and structural identification to carthamin by a purified enzyme is yet to be demonstrated.

In this research, precarthamin was isolated efficiently from the immature petals of safflower and identified with various NMR techniques. The isolated precarthamin was converted to carthamin by a homogeneously purified enzyme from the immature petals of safflower in vitro.

EXPERIMENTAL PROCEDURES

Materials. Sephadex LH-20, Sephadex G-25 and DEAE Sepharose were bought from Pharmacia Biotech. Bio-Gel A-0.5 m and Macro-Prep Ceramic Hydroxyapatite were purchased from Bio-Rad Laboratories. Solvents for HPLC were from Merck and other organic solvents were from Hayman and Tedia. Ammonium sulfate and citrate were from Amresco and Duchefa, respectively. Other chemicals and DMSO-d_6 used as NMR solvent were obtained from Sigma and Aldrich, respectively. The immature yellow petals of safflower were harvested at 1−2 days after blooming and stored at −70 °C until use.

Extraction and Isolation of Precarthamin. Precarthamin was isolated from the yellow petals of safflower by the method of Kazuma et al. (1995) with minor modification. The yellow petals of safflower (200 g) were extracted with 2 L of MeOH for 24 h to remove yellow pigments. After filtration, the filtered petals were homogenized and extracted with 400 mL of acetone containing 1% TFA. The extract was concentrated with rotary evaporator and the concentrate was filtered through Whatman no. 2 filter paper to remove solid materials. The filtrate was subjected to the Sephadex LH-20 column and eluted with linear gradient of 20 to 80% CH_3CN/H_2O containing 1% TFA. The precarthamin fractions were concentrated and further isolated with preparative reversed-phase HPLC (Megapac Sil C18-10 column, eluted with CH_3CN/MeOH/TFA/H_2O = 30/10/1/59). A preparative HPLC yielded 50 mg of precarthamin (**1**). The isolated precarthamin was iden-

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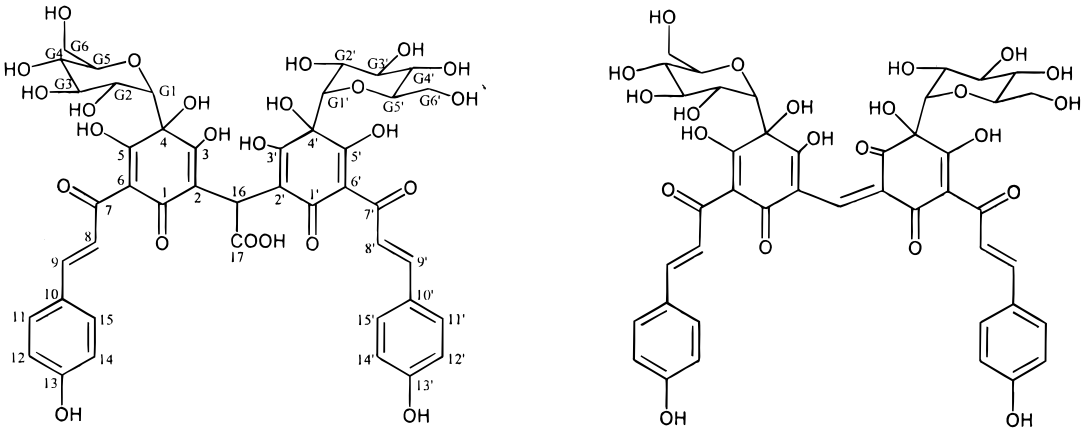

1 2

Figure 1. Chemical structures of precarthamin (**1**) and carthamin (**2**).

tified with NMR spectrometry. The ^1H and ^{13}C NMR data of the isolated pigment were obtained with 400 MHz FT-NMR (JEOL) spectrometer at 400 and 100 MHz, respectively.

Isolation of Carthamin. Carthamin, a red coloring matter of safflower, was isolated by the method of Kim et al. (1996) with minor modification. The dried petals of safflower were washed with H_2O and MeOH to remove yellow pigments. The washed petals were extracted with 0.1 M K_2CO_3 and then acidified with 0.5 M citric acid to precipitate carthamin. Carthamin precipitate was dissolved in MeOH and then applied to Sephadex LH-20 column. Sephadex LH-20 column was eluted with MeOH, and carthamin fractions were collected. By comparing the NMR data with those of carthamin reported by Kim et al. (1996), we identified the isolated pigment as carthamin (**2**) and used as an authentic sample.

Enzyme Activity Assay. Precarthamin decarboxylase catalyzes the oxidative decarboxylation reaction of precarthamin to carthamin (Figure 1). An enzyme activity was measured by monitoring the change of A_{520} (Figure 2) with UV−vis spectrophotometer (Varian, DMS-300) using the difference of λ_{max} values between precarthamin (λ_{max} = 406 nm) and carthamin (λ_{max} = 520 nm). A reaction mixture contains 50 mM citrate buffer, pH 5.0, precarthamin (20 μM), and an enzyme preparation. An enzyme reaction was carried out at 25 °C.

Purification of Precarthamin Decarboxylase. An enzyme catalyzing the oxidative decarboxylation of precarthamin to carthamin was purified homogeneously by following procedure from the yellow petals of safflower. Unless otherwise stated, all of the operation was performed at 4 °C. The yellow petals of safflower (1 kg) were homogenized with 3.2 L of 50 mM Tris buffer (pH 7.5). The homogenate was filtered through nylon cloth and clarified with centrifugation. The clarified sample was precipitated between 20 and 60% saturation of ammonium sulfate. After centrifugation, the pellet was dissolved in 500 mL of 50 mM Tris buffer (pH 7.5), and second ammonium sulfate fractionation between 20 and 50% saturation of ammonium sulfate. After centrifugation, the pellet was dissolved in 32 mL of 50 mM Tris buffer (pH 7.5). The sample obtained from second ammonium sulfate fractionation was desalted with Sephadex G-25 column chromatography. The desalted sample was applied to the DEAE Sepharose column (2.5 × 23 cm) equilibrated with 50 mM Tris buffer (pH 7.5), and washed with the same buffer. The enzyme activity was appeared in washing fractions. The active fractions were pooled and precipitated with 80% saturation of ammonium sulfate. After centrifugation, the pellet was dissolved in 10 mL of 10 mM sodium phosphate buffer (pH 7.0) and desalted with Sephadex G-25 column chromatography. The desalted sample was subjected to the Macro-Prep Ceramic Hydroxyapatite column (2.5 × 3 cm) equilibrated with 10 mM sodium phosphate buffer (pH 7.0). After washing with the same buffer, the sample was eluted with linear gradient of 10 to 200 mM sodium phosphate buffer (pH 7.0). The active fractions were pooled and concentrated with 80% saturation of ammonium sulfate. The concentrate was applied to the Bio-Gel A-0.5 m column (2.5 × 105 cm) equilibrated with 50 mM Tris buffer (pH 7.5) containing 50 mM NaCl, and eluted with the same buffer. The active fractions were pooled and precipitated with 80% saturation of ammonium sulfate. After centrifugation, the pellet was dissolved in 10 mL of 10 mM sodium phosphate buffer. The sample was subjected to Macro-Prep Ceramic Hydroxyapatite column (2.5 × 3 cm) equilibrated with 10 mM sodium phosphate buffer, pH 7.0. After washing with the same buffer, the sample was eluted gradiently with 10 to 100 mM sodium phosphate buffer, pH 7.0. The active fractions were pooled and used as an enzyme for the conversion of precarthamin to carthamin.

Electrophoretic Analysis. SDS−PAGE was performed according to the method of Laemmli (1970) in 12% acrylamide running gel. Proteins were stained with Coomassie brilliant blue staining solution.

RESULTS AND DISCUSSION

Isolation and Identification of Precarthamin. The flower color of safflower (*Carthamus tinctorius* L.) gradually changes from yellow to red, which is due to the synthesis of carthamin from the yellow precursors. Precarthamin was thought to be a direct precursor of carthamin (Saito et al., 1983a,b, 1985; Kumazawa et al., 1994; Kazuma et al., 1995). Because of its instability, the isolation of precarthamin was difficult and the yield was low. Kumazawa et al. (1994) and Kazuma et al. (1995) isolated precarthamin and determined the chemi-

Table 1. ^{1}H and ^{13}C NMR Data of Precarthamin from the Yellow Petals of Safflower (in DMSO-d_6, chemical shifts in parts per million)

position[a]	^{1}H NMR	^{13}C NMR
1, 1′		193.69, 193.58
2, 2′		107.02, 106.67
3, 3′		174.04, 173.47
4, 4′		81.46, 81.29
5, 5′		172.66
6, 6′		107.74
7, 7′		178.64, 178.56
8, 8′	7.30 (*d*, *J* = 16 Hz, 1H)	118.91
	7.28 (*d*, *J* = 16 Hz, 1H)	
9, 9′	7.58 (*d*, *J* = 16 Hz, 2H)	140.60, 140.40
10		126.35
11, 11′, 15, 15′	7.52 (*d*, *J* = 8.8 Hz, 2H)	130.45
	7.50 (*d*, *J* = 8.8 Hz, 2H)	
12, 12′, 14, 14′	6.83 (*d*, *J* = 8.8 Hz, 2H)	115.93
	6.80 (*d*, *J* = 8.8 Hz, 2H)	
13, 13′		159.83, 159.78
16	4.82 (*s*, 1H)	36.55
17		189.81
G1, G1′	3.50 (*d*, *J* = 9.6 Hz, 2H)	86.84, 86.46
G2, G2′	3.33 (*m*, 2H)	68.87
G3, G3′	3.13 (*m*, 2H)	68.53, 68.13
G4, G4′	2.88 (*m*, 2H)	79.73, 79.47
G5, G5′	3.11 (*m*, 2H)	78.48
G6, G6′	3.45 (*m*, 2H)	60.05
	3.30 (*m*, 2H)	59.65

[a] The positions are shown in Figure.

cal structure. They obtained 100 mg (Kumazawa et al., 1994) and 30 mg (Kazuma et al., 1995) of precarthamin from 10 kg and 670 g of the petals of safflower, respectively. In this research, we isolated precarthamin efficiently with Sephadex LH-20 column chromatography and preparative HPLC. 200 g of the fresh yellow petals of safflower yielded 50 mg of precarthamin, which is much higher yield compared with those of previously reported (Kumazawa et al., 1994; Kazuma et al., 1995).

The isolated pigment was identified as precarthamin with various NMR techniques and UV−vis spectrophotometer (Figure 1). The UV−vis spectrum of the pigment showed λ_{max} of 406 and 238 nm in MeOH. The molar extinction coefficients of the pigment in MeOH were 59 000 M^{-1} cm^{-1} at 406 nm and 24 000 M^{-1} cm^{-1} at 238 nm. The molar extinction coefficient of the pigment in 50 mM citrate buffer (pH 5.0) was 45 400 M^{-1} cm^{-1} at 406 nm. These values are similar to those of the other yellow pigments of safflower (Onodera et al., 1981; Takahashi et al., 1984; Onodera et al., 1989), which suggest that the pigment has chalcone moeity. The molar extinction coefficients of precarthamin in MeOH was slightly higher than those of the values (46 400 M^{-1} cm^{-1} at 406 nm and 23 600 M^{-1} cm^{-1} at 238 nm) reported by Kazuma et al. (1995). This difference was may be due to the purity of precathamin. The ^{1}H NMR spectrum showed typical proton peaks of two chalcone moeities at δ 6.80−7.58 and peaks of two glucose moieties at δ 3.11−3.50. Kumazawa et al. (1994) and Kazuma et al. (1995) assigned differently the signal of methine proton (H-16) at δ 5.42 in DMSO-d_6 and δ 7.03 in pyridine-d_5/methanol-d_4 (95/4), respectively. By analysis of ^{1}H-^{13}C COSY and HMQC spectra, we assigned the methine proton (H-16) and carbon (C-16) signals at δ 4.82 (*s*, 1H) and δ 36.55 in DMSO-d_6, respectively. The ^{1}H and ^{13}C NMR data of precarthamin were summarized in Table 1.

Purification of Precarthamin Decarboxylase. Precarthamin decarboxylase catalyzing oxidative decarboxylation of precarthamin to carthamin was homo-

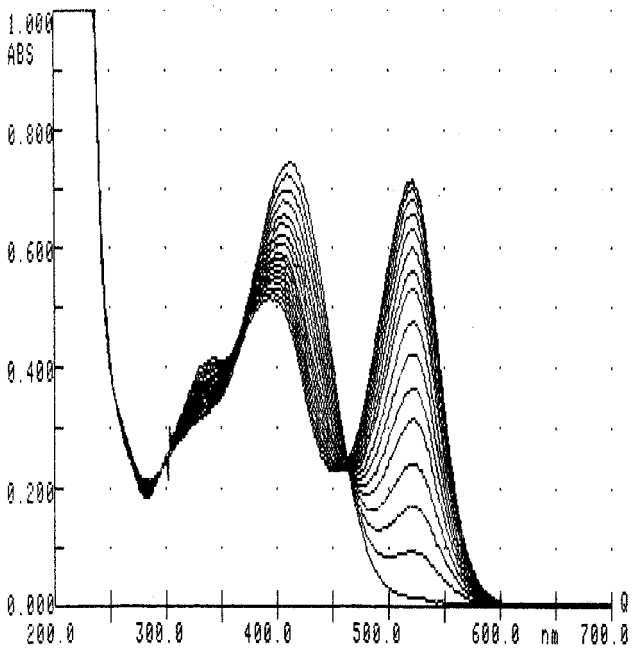

Figure 2. UV−vis spectral changes during the enzymatic conversion of precarthamin to carthamin. The UV−vis spectra were measured at 5 min interval after mixing the enzyme preparation and precarthamin in 50 mM citrate buffer (pH 5.0).

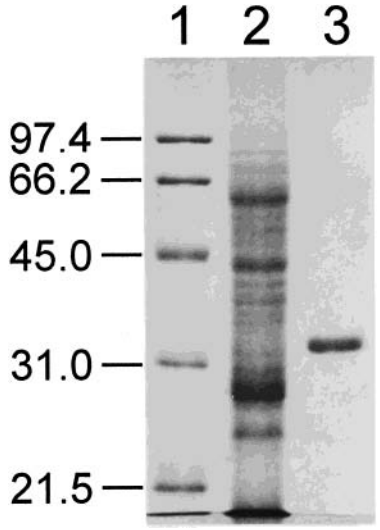

Figure 3. SDS−PAGE of the purified carthamin synthesizing enzyme. Protein samples were from after crude extraction (2) and second Macro-Prep Ceramic Hydroxyapatite column chromatography (3). Size marker proteins (1) were phosphorylase b (97.4 kDa), BSA (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa).

geneously purified to 12600-folds with 4.54% yield by two times of ammonium sulfate fractionation and sequential chromatographic techniques, including DEAE Sepharose, Macro-Prep Ceramic Hydroxyapatite and Bio-Gel A-0.5 m column chromatography from the yellow petals of safflower (Figure 3). The monomeric molecular weight of the enzyme was determined as 33 000 by SDS−PAGE (Figure 3). To determine the optimum pH of the enzyme reaction, the enzyme activity was measured at pH ranges from 3.0 to 9.0. The enzyme was inactive at pH below 4.0. The enzyme activity sharply increased with increase of pH more than 4.0 and the activity was maximum at pH 5.0. The enzyme

Table 2. UV−Vis Spectral and TLC Data of Enzymatically Converted and Authentic Carthamins

	λ_{max} in MeOH	R_f value (%)		
		BAW	BEW	BTPW
enzymatically converted	373, 516	39.6	57.0	67.7
authentic	372, 518	39.9	57.3	67.9

[a] Solvents used: BAW (BuOH:AcOH:H_2O = 4:1:5, upper layer), BEW (BuOH:EtOH:H_2O = 4:1:2), BTPW (BuOH:toluene:pyridine:H_2O = 5:1:3:3, upper layer).

activity decreased with increase of pH ranges from 5.0 to 7.0 (Cho and Hahn, 2000). This result is in good agreement with the optimum pH reported previously (Saito et al., 1983a,b). The saturation curve of the enzyme to precarthamin shows hyperbolic shape, indicating that the enzyme follows Michaelis−Menten kinetics. The Michaelis constant (K_m) and V_{max} of the enzyme to precarthamin was 164 μM and 29.2 nmol/min, respectively. The turnover number (k_{cat}) of the enzyme was calculated as 1.42×10^2 s^{-1} (Cho and Hahn, 2000). The K_m values of some decarboxylases are in the range from 10^{-3} to 10^{-5} M (Hayasaka et al., 1980; Ng et al., 1982; Phan et al., 1983; Baetz and Allison, 1989; Shigeoka and Nakano, 1991; Xu et al., 1996; Nam et al., 1997; Kim et al., 1998). The K_m of precarthamin decarboxylase was 1.64×10^{-4} M, indicating that the enzyme has a relatively high affinity to precarthamin. The turnover number (k_{cat}) of precarthamin decarboxylase was 1.42×10^2 s^{-1}. The k_{cat}/K_m value of the enzyme was 8.7×10^5 M^{-1} s^{-1}, indicating that enzymatic efficiency of the conversion of precarthamin to carthamin by precarthamin decarboxylase is relatively high. The detailed biochemical characteristics of the purified enzyme is reported elsewhere (Cho and Hahn, 2000).

Conversion of precarthamin to carthamin in vitro by a purified enzyme. The last step of color transition of safflower was conversion of precarthamin to a red pigment, carthamin. Saito et al. (1983a,b; 1985) isolated a putative precursor of carthamin and converted to carthamin by enzyme preparations obtained from vegetative tissues and seedlings of safflower. In this research, we purified an enzyme, precarthamin decarboxylase, and converted precarthamin to carthamin using the purified enzyme. Since the purified enzyme showed maximum activity at pH 5.0 in 50 mM citrate buffer, the enzymatic conversion of precarthamin to carthamin was carried out in 50 mM citrate buffer (pH 5.0). The isolated precarthamin was converted by addition of the purified enzyme in 50 mM citrate buffer, pH 5.0, to carthamin. The UV−vis spectral changes during the conversion reaction (Figure 2) showed gradual decrease of A_{406} ($\lambda_{vis,max}$ of precarthamin) and increase of A_{520} ($\lambda_{vis,max}$ of carthamin). After the reaction completed, the reaction mixture was fractionated with BuOH to obtain the enzymatically converted red pigment. The UV−vis spectrum of the red pigment showed 373 and 516 nm of λ_{max} in MeOH (Table 2), which was in good agreement with that of authentic carthamin (Figure 4). The R_f values of the red pigment on TLC with different solvent systems were essentially identical with those of authentic carthamin (Table 2). The pigment also showed the same retention time in HPLC analysis as authentic carthamin (Figure 5). Therefore, the red pigment enzymatically converted from precarthamin was identified as carthamin.

In summary, we isolated precarthamin efficiently from the immature yellow petals of safflower with simple procedures of Sephadex LH-20 column chromatography and preparative HPLC. An enzyme involved in the last step of carthamin biosynthesis was homogeneously purified and named precarthamin decarboxylase. Precarthamin was converted to a red pigment by a homogeneously purified enzyme from the yellow petals of safflower in 50 mM citrate buffer (pH 5.0), and the red pigment was identified as carthamin.

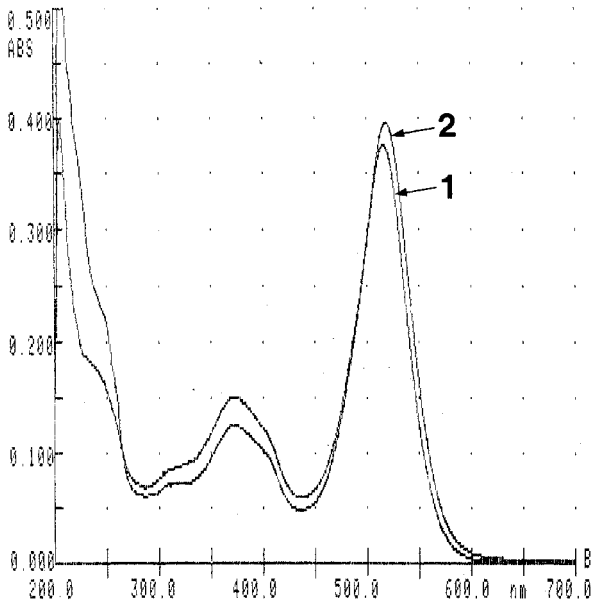

Figure 4. UV−vis spectra of the enzymatically converted (1) and authentic (2) carthamins in MeOH.

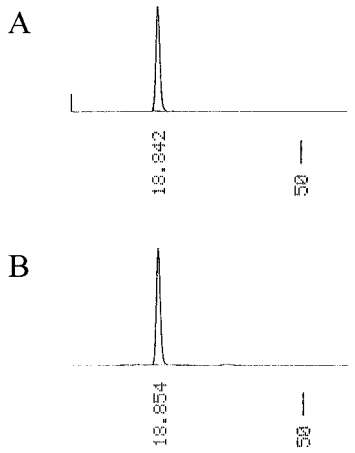

Figure 5. HPLC of enzymatically synthesized (A) and authentic (B) carthamins. The samples were analyzed by reversed-phase HPLC (Megapac Sil C18-10 column, eluted with CH_3CN/MeOH/TFA/H_2O = 30/10/1/59).

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