HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF CARTHAMIN, SAFFLOR YELLOW A AND A PRECURSOR OF CARTHAMIN

APPLICATION TO THE INVESTIGATION OF AN UNKNOWN RED PIGMENT PRODUCED IN CULTURED CELLS OF SAFFLOWER

KATSUYUKI NAKANO*

PL Comprehensive Research Institute, 1 Kamiyamacho, Tondabayashi, Osaka 584 (Japan)

YOSHIHIRO SEKINO, NAOGORO YOMO, SACHIO WAKAYAMA, SHUJI MIYANO, KAZUHITO KUSAKA and ETSUKO DAIMON

Kibun Co., 7-14-13 Ginza, Chuou-ku, Tokyo 104 (Japan)

KAZUMITSU IMAIZUMI, YOSHINOBU TOTSUKA and SHIGERU ODA

PL Botanical Institute, 1 Kamiyamacho, Tondabayashi, Osaka 584 (Japan)

and

YASUYUKI YAMADA

Research Center for Cell and Tissue Culture, Kyoto University, Sakyo-ku, Kyoto 606 (Japan)

(First received October 2nd, 1987; revised manuscript received November 30th, 1987)

SUMMARY

A reversed-phase high-performance liquid chromatographic (RP-HPLC) method was developed to analyse and purify carthamin, safflor yellow A (safflomin-A) and the yellow precursor of carthamin. A red pigment similar to carthamin was extracted from cell suspension cultures of safflower (*Carthamus tinctorius* L.) as an application of biotechnology. The RP-HPLC method was used to characterize the unknown red pigment. Various spectroscopic methods were used to characterize purified carthamin and the purified unknown pigment. Based on these spectral observations, it was concluded that the unknown red pigment produced in cultured cells of safflower differed from carthamin, although the unknown pigment might be a glycoside. From other observations, the possibility of anthocyanins and carotenoids were also discounted. It was assumed that the cultured cells lacked the biosynthetic pathway of the yellow precursor of carthamin, although they possessed the activity of the enzyme mediating the synthesis of carthamin from the yellow precursor.

INTRODUCTION

In recent years, natural pigments have been looked at again from the point of safety, especially in the field of food hygiene chemistry and cosmetic chemistry. Carthamin, the red colouring matter of the petals of safflower (*Carthamus tinctorius* L.) is a natural plant pigment, and has long been used in cosmetics, textile dyestuffs and herb medicine. Safflower oil abundant in linoleic acid is obtained from the seeds of this plant.

Recently, the structure of carthamin has been re-examined by Obara and On-odera[1], and Takahashi et al.[2] and proposed to be as follows instead of those assigned previously[3,4]: 1-(2,6-diketo-3-glucosyl-3,4-dihydroxy-5-p-hydroxycinnamoyl)cyclo-hex-4-enylidene-1'-(2',3',4'-trihydroxy-3'-glucosyl-5'-p-hydroxycinnamoyl-6'-keto)-cyclohexa-1',4'-dienylmethane[1].

Carthamin has hitherto been extracted only from flowers of safflower, and is thus very expensive. Therefore, we have developed methods to produce and extract the red pigment in cultured cells of safflower as an application of biotechnology. It is interesting to determine whether this red pigment is identical to carthamin.

To analyse and purify carthamin, open-column chromatography and thin-layer chromatography (TLC) have been used[5,6]. However, there is no information on high-performance liquid chromatographic (HPLC) methods to analyse carthamin.

Recently, an enzyme which catalysed the biosynthesis of carthamin from the yellow precursor of carthamin was found in the vegetative tissues of safflower[5,6].

In this paper, we describe a reversed-phase HPLC (RP-HPLC) method to ana-lyse and isolate carthamin, safflor yellow A (safflomin-A), the yellow pigment of the carthamin precursor, and the unknown red pigment from cultured cells of safflower. We report the results of ultraviolet–visible (UV–VIS), infrared (IR), ^{1}H nuclear mag-netic resonance (NMR) and mass spectroscopic (MS) measurements on carthamin and an unknown red pigment. The similarities and differences between the unknown pigment from cultured cells and carthamin are discussed. The activity of the enzyme mediating the synthesis of carthamin from its precursor has been examined using homogenates of cultured cells of safflower.

EXPERIMENTAL

Chemicals and standards

Methanol (HPLC grade), acetone (reagent grade) and pyridine (reagent grade) were obtained from Wako (Osaka, Japan). Cellulose powder was obtained from Asahi Kasei (Tokyo, Japan).

A standard sample of carthamin purified from flowers of safflower was a gift from Sanei Chemical (Osaka, Japan). A 7-mg amount of carthamin was dissolved in 50 ml of methanol for the analytical RP-HPLC, and concentrated with a rotary evaporator for the preparative RP-HPLC.

Apparatus

A Shimadzu LC-6A (system controller and two pumps), SPD-6AV UV–VIS spectrophotometric detector and a C-R3A Chromatopac data processor were used for HPLC (Shimadzu, Kyoto, Japan).

UV aborption spectra of fractionated HPLC peaks were measured with a Model 200-10 spectrophotometer (Hitachi, Tokyo, Japan). Electron impact (EI) mass spectra and ^{1}H NMR spectra in [^{2}H$_5$]pyridine were measured with Shimadzu GC-MS 9000 and JEOL GX-400 (400 MHz) instruments at Kyoto University. ^{1}H and ^{13}C NMR spectra in [^{2}H$_6$]dimethyl sulphoxide were measured with a VXR-300 (300 MHz) instrument (Varian Instruments Ltd., Tokyo, Japan). FT-IR spectra were measured with a JEOL JIR-100 instrument at the Toray Research Center.

Chromatographic conditions

Reversed-phase analytical columns (250 mm × 4.6 mm I.D.) or a preparative column (250 mm × 10 mm I.D.) packed with 5-μm Develosil ODS-5 (Nomura, Nagoya, Japan) and a pre-column (50 mm × 4.0 mm I.D.; Chemuco, Osaka, Japan) packed with 15–30 μm Develosil ODS were used. A filter (mesh size 1 μm; Nomura) was fitted between the injector and the pre-column.

For the analytical separation of the solution of carthamin and the unknown pigment from cultured cells, linear gradient elution from methanol–water (40:60) to methanol–water (80:20) in 15 min was performed at the flow-rate of 1 ml/min. For the preparative separation of the carthamin standard, isocratic elution was used with methanol–water (70:30) at the flow-rate of 1.3 ml/min. For the preparative separation of the solution of the unknown pigment, linear gradient elution from methanol–water (65:35) to methanol–water (90:10) in 15 min was used at the flow-rate of 1.8 ml/min.

The column temperature was ambient. All eluents were degassed by purging with helium. The detection was performed at 254 or 520 nm.

For the separation of safflor yellow A and the yellow precursor of carthamin, linear gradient elution from methanol–water (10:90) to methanol–water (60:40) in 15 min was used at the flow-rate of 1 ml/min. The detection was performed at 410 nm (0.04 a.u.f.s.).

Extraction of the unknown pigment from cultured cells of safflower

The tissues of flower buds from safflower before and after blossoming were separated using a scalpel under a microscope and sterile conditions. The tissues were cultivated on agar containing the modified Murashige-Skoog medium[7] until callus was formed. The callus was transferred to a liquid medium with the same chemical composition as the above for suspension culture on a rotary shaker (120 rpm). After the cultured cells had proliferated sufficiently, allowing repeated transfers to new medium, cellulose powder (or filter-paper) was added to the culture medium. After a few days, the colour of the cellulose powder had turned red. The red powder was collected by filtration of the culture medium with a nylon sieve, and dried at room temperature. The details of these procedures for tissue cultures will be published elsewhere.

Extraction of the unknown pigment from cellulose powder

The unknown red pigment was extracted by adding five volumes (per gram of cellulose) of pyridine or acetone–methanol (80:20) to from 24 to 100 g, of the red cellulose powder, for analytical and preparative experiments. The mixture was centrifuged at 1500 g for 5 min. The red supernatant was collected, and the extraction repeated once more for pyridine or three times more for acetone–methanol.

The unknown red pigment was extracted from cellulose powder strongly by pyridine and weakly by acetone–methanol. The extraction of the unknown pigment from cultured cells is very similar to that of carthamin. However, ^{1}H NMR measurements were reported[8] which showed that crystals of carthamin contain pyridine due to the presence of pyridine in the extraction processes, so we selected acetone–methanol as an extraction solvent for the preparative analysis of the unknown pigment so as to avoid the interference by pyridine with the identification of the pigment.

The solution of the unknown red pigment extracted by pyridine was directly

analysed by RP-HPLC. For the preparative analysis, the solution of the unknown pigment extracted by acetone–methanol was concentrated to about 10 ml with a rotary evaporator at less than 30°C. The concentrated pigment solution was centrifuged again at 1500 g for 5 min to remove the white and soft matter, which was presumed to be lipids from cultured cells, and the supernatant was analysed by preparative RP-HPLC.

Crystallization of carthamin and the purified unknown pigment from cultured cells

Carthamin and the unknown red pigment from cultured cells in methanol–water, which were fractionated by RP-HPLC, were concentrated with a rotary evaporator at less than 30°C, until an aqueous solution of 2–3 ml was obtained.

The unknown pigment extracted from cellulose is less thermally stable in water than is carthamin and is degraded irreversibly to a light yellow compound at room temperature and in the presence of light within a few days. Upon crystallization under reduced pressure, even at 4 or −20°C, the unknown pigment formed a brown amorphous substance and a few purple-blue crystals.

Finally, solutions of carthamin and the purified unknown red pigment were lyophilized immediately after concentration, forming brick-red and purple-blue crystals, respectively. The apparent colour of the unknown pigment changed from red to purple-blue during the concentration of its water solution for lyophilization. When the lyophilized unknown pigment was redissolved in methanol, its apparent colour became red again. The maximum yield of crystals of the unknown pigment from cultured cells was about 5 mg from 26.9 g of cellulose powder, although this yield changed depending on the ability of the cultured cells to alter the colour of cellulose powder.

Extraction of the yellow precursor of carthamin from flower petals of safflower

The flower buds of safflower before full blossom were frozen in a freezer at −20°C until use. A 2.8-g amount of yellow petals was homogenized with 50 ml of methanol. The methanol extraction was repeated four times. A quarter volume of water was added to the pooled methanol extracts, and concentrated using a rotary evaporator to remove methanol. The concentrated pigment solution was filtered, and frozen at −20°C until use.

By RP-HPLC analyses of the concentrated pigment solutions, it was found that the solutions contained two major yellow pigments, safflor yellow A or safflomin-A (peak A in HPLC), and the yellow precursor of carthamin (peak B in HPLC). The precursor of carthamin was fractionated by RP-HPLC.

Furthermore, the yellow precursor of carthamin was also isolated most easily by using a Sep-Pak C_{18} cartridge (Waters Assoc., Milford, MA, U.S.A.). When 2 ml of the concentrated pigment solution were applied to a Sep-Pak, all pigments were adsorbed. One, safflor yellow A (peak A in HPLC), was eluted with four volumes of methanol–water (10:90) (recovery: 93.4% by HPLC analysis), and the yellow precursor of carthamin (peak B in HPLC) was eluted with methanol–water (30:70) (recovery: 87.7% by HPLC). The separation of two yellow pigments was almost complete from the results of RP-HPLC analysis of each Sep-Pak elute. Finally, carthamin was eluted with two volumes of more than 60% methanol in water.

RESULTS

Analytical chromatography of carthamin and the unknown pigment from cultured cells

The chromatograms in Fig. 1 show the difference in retention behaviour in analytical RP-HPLC for a standard sample of carthamin and the unknown red pigment from cultured cells. The red pigment extracted with pyridine from the petals of safflower showed the same retention time as that of the carthamin standard. The unknown pigment from cultured cells is presumed to be more hydrophobic than carthamin.

As seen in Fig. 1b, the solution of the unknown pigment from cultured cells showed only one major peak upon detection at 520 nm, indicating that the solution contained only one red compound. However, the pigment solution from cultured cells contained many other UV-absorbing compounds, as shown later. The existence of only one red compound was confirmed by fractionation of other UV-absorbing peaks.

Preparative chromatography of carthamin and the unknown pigment from cultured cells

The carthamin standard in methanol was purified using preparative RP-HPLC with isocratic elution as shown in Fig. 2. The chromatogram indicates that this standard contains several other UV-absorbing compounds.

To identify an unknown red pigment extracted from cultured cells, the red peak of the concentrated pigment solution was fractionated by preparative RP-HPLC with linear gradient elution as shown in Fig. 3a. The chromatogram in Fig. 3b shows an HPLC separation of the fractionated peak, indicating the purity of the isolated
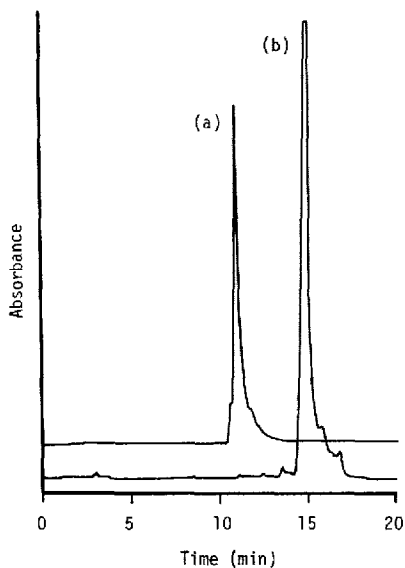

Fig. 1. Chromatograms of a carthamin standard (a) and of the unknown pigment from cultured cells of safflower (b). Column: Develosil ODS-5 (5 μm, 250 mm × 4.6 mm I.D., Nomura). Eluents: A, water; B, methanol; linear gradient from 40 to 80% B in 15 min; flow-rate, 1 ml/min. Detection: 520 nm (0.04 a.u.f.s.). Temperature: ambient. Injection volumes: 10 μl (a) and 50 μl (b).

red pigment, and the elimination of the greater part of the other UV-absorbing compounds.

UV–VIS absorption spectra of purified carthamin and of the purified unknown pigment from cultured cells

Fig. 4a and b show the UV–VIS absorption spectra of purified carthamin and of the purified unknown pigment from cultured cells in methanol–water. Although the spectrum of carthamin with a relatively broad main peak at around 522 nm and a second peak at around 370 nm coincided exactly with that given previously[1], the spectrum of the unknown pigment exhibited a sub-peak around 490 nm besides a relatively sharp main peak at around 523 nm, differing from that of carthamin.

The solution of the crude pigment extracted from cellulose powder showed the same visible spectrum as seen in Fig. 4b, indicating that the unknown pigment was not influenced by the purification processes.

IR spectra of crystals of purified carthamin and of the purified unknown pigment from cultured cells

The spectra in Fig. 5a and b show the results of Fourier transform IR measurements in potassium bromide pellets of purified carthamin and the purified unknown pigment. Our IR spectrum for carthamin coincided exactly with that given previously[5]. However, the IR spectrum of the unknown pigment from cultured cells was significantly different from that of carthamin, except for the presence of the
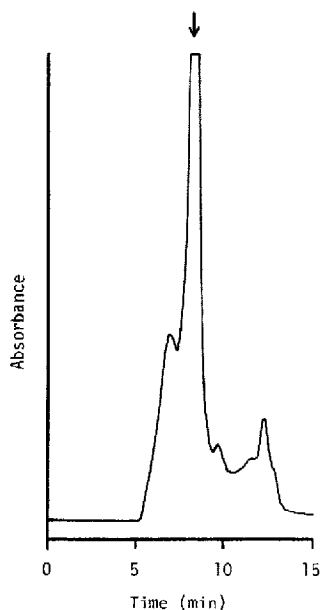

Fig. 2. Preparative RP-HPLC separation of the carthamin standard. Column: Develosil ODS-5 (5 μm, 250 mm × 10 mm I.D.). Eluents: A, water; B, methanol; isocratic elution with 70% B; flow-rate, 1.3 ml/min. Detection: UV at 254 nm (0.64 a.u.f.s.). Temperature: ambient. Injection volume: 150 μl. An arrow shows the peak of carthamin.
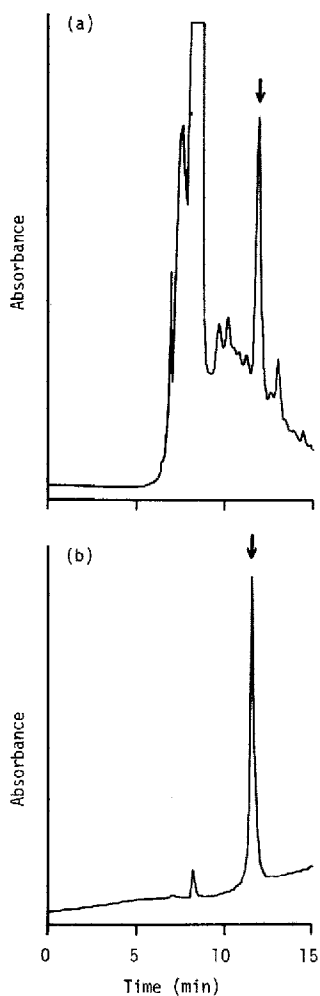
Fig. 3. Preparative RP-HPLC separation of the unknown pigment from cultured cells of safflower (a) and the fractionated unknown pigment (b). Column: Develosil ODS-5 (5 μm, 250 mm × 10 mm I.D.). Eluents: A, water; B, methanol; linear gradient from 65 to 90% B in 15 min; flow-rate, 1.8 ml/min. Detection: UV at 254 nm; 2.56 a.u.f.s. (a) and 0.04 a.u.f.s. (b). Temperature: ambient. Injection volumes: 450 μl (a) and 20 μl (b). The arrows show the peaks of the unknown pigment.

hydroxyl groups of the glucose moiety inferred by the strong broad absorption between 3300 and 3500 cm^{-1}.

MS spectra of purified carthamin and of the purified unknown pigment from cultured cells

First, EI-MS measurements on purified carthamin and the purified unknown pigment were tried. However, both pigments were not vaporized by elevation of the temperature to 300°C. Field desorption mass spectra of carthamin showed the abun-
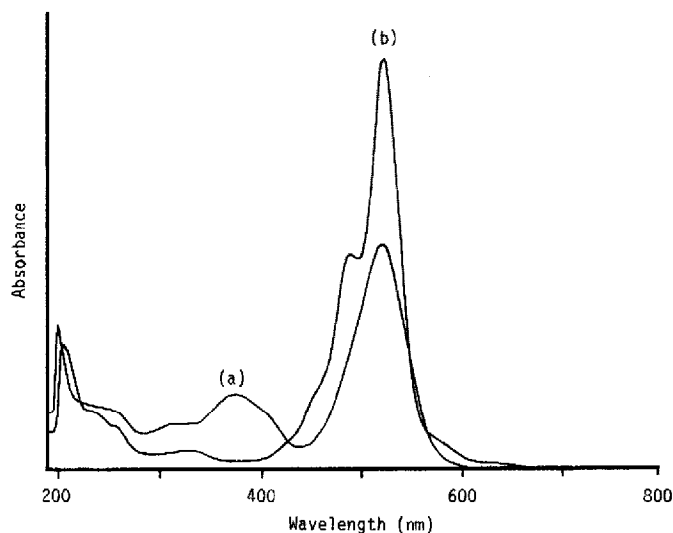
Fig. 4. UV absorption spectra of purified carthamin (a) and of the purified unknown pigment from cultured cells of safflower (b) in methanol–water.

dant fragment ion peaks at m/e 363 and 568 (ionization: cathode voltage, 4.5 kV; wire current, 1.0 mA/min) or 120 and 325 (cathode voltage, 5.5 kV; wire current, 2.5 mA/min). The peak at m/e 568 corresponds to a fragment produced by the loss of one molecular water from the aglycone of carthamin, as indicated previously[1]. Next, field desorption and fast atom bombardment mass spectra gave the most abundant fragment ion peak at m/e 358 for the unknown pigment from cultured cells. The results also indicated that the unknown pigment differed from carthamin.

NMR spectra of purified carthamin and of the purified unknown pigment from cultured cells

  ^{1}H NMR measurements were performed on purified carthamin and the purified unknown pigment from cultured cells in [^{2}H$_5$]pyridine at 400 MHz. In the ^{1}H NMR spectrum of carthamin, the following signals were observed: glucosyl protons (3.26–5.00 ppm, m), *para*-substituted phenyl protons (6.90 and 7.50 ppm, 4H, d, $J = 8.5$ Hz), *trans*-vinylene protons (8.08 and 8.33 ppm, 2H, d, $J = 15.9$ Hz), a proton of one methine group (9.40 ppm, 1H, s), phenolic protons (12.10 ppm, 2H, s) and enol protons (19.83 ppm, 2H, s). These results are in good agreement with those from other measurements in [^{2}H$_6$]dimethyl sulphoxide[1] except for a slight shift to lower magnetic field due to the use of the different solvent.

  However, the concentrated solution of the unknown red pigment in [^{2}H$_5$]pyridine formed a precipitate. As shown in Fig. 6, the ^{1}H NMR spectrum of the unknown pigment in [^{2}H$_6$]dimethyl sulphoxide differed remarkably from that of carthamin, except for the presence of glucose-like signals (4.0–5.3 ppm in Fig. 6) as suggested from the IR measurement. The ^{1}H NMR spectrum of the unknown pigment completely lacked proton signals from an aromatic chromophore. The ^{13}C NMR spectrum also showed the presence of glucose-like CH signals and the lack of
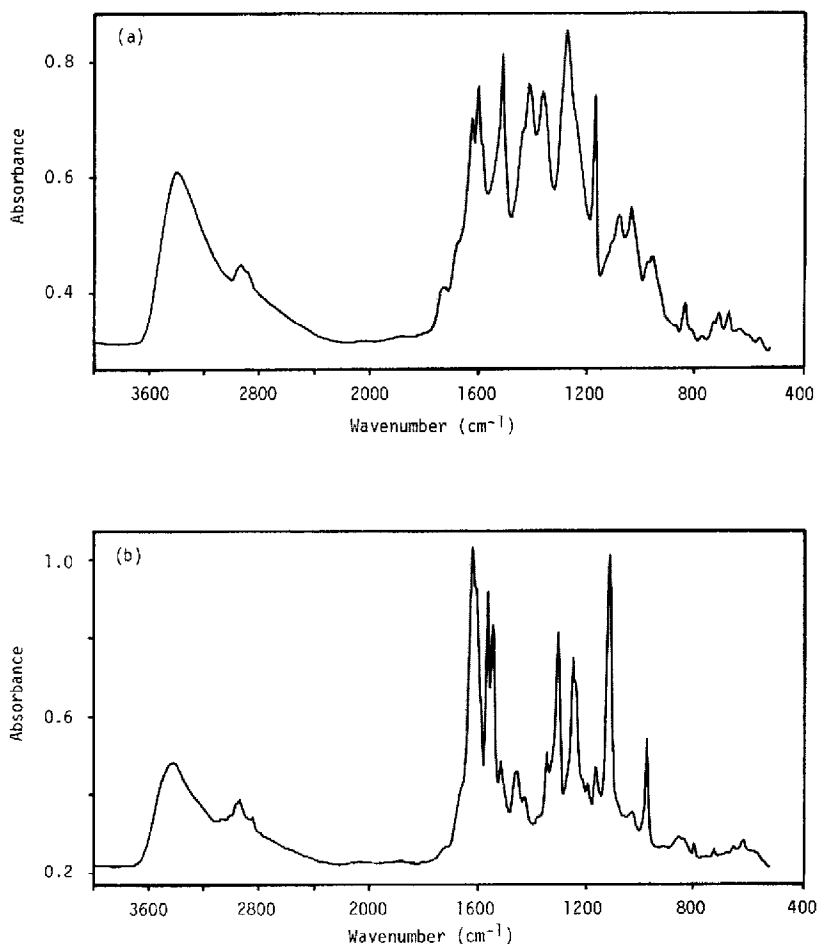
Fig. 5. IR spectra in potassium bromide pellets of purified carthamin (a) and of the purified unknown pigment from cultured cells of safflower (b).

carbon signals from an aromatic chromophore. It was inferred that the chromophore of the unknown pigment from cultured cells had been degraded during the purification processes, because of its instability, to leave an undetectable amount.

HPLC separation of safflor yellow A and the yellow precursor of carthamin

      The chromatogram in Fig. 7 shows the RP-HPLC separation of the pigment solution extracted from petals of safflower. Two major peaks due to yellow pigments (peaks A and B in Fig. 7) were fractionated, and their UV–VIS absorption spectra were measured. Peak A exhibited maximum absorption around 403 nm and a shoulder at around 340 nm. The maximum was coincident with that of safflor yellow A[2] (or safflomin-A[9]). Peak B showed maximum absorption around 417 nm and a shoulder at around 340 nm. The absorption maximum was identical with that of the precursor of carthamin[6].
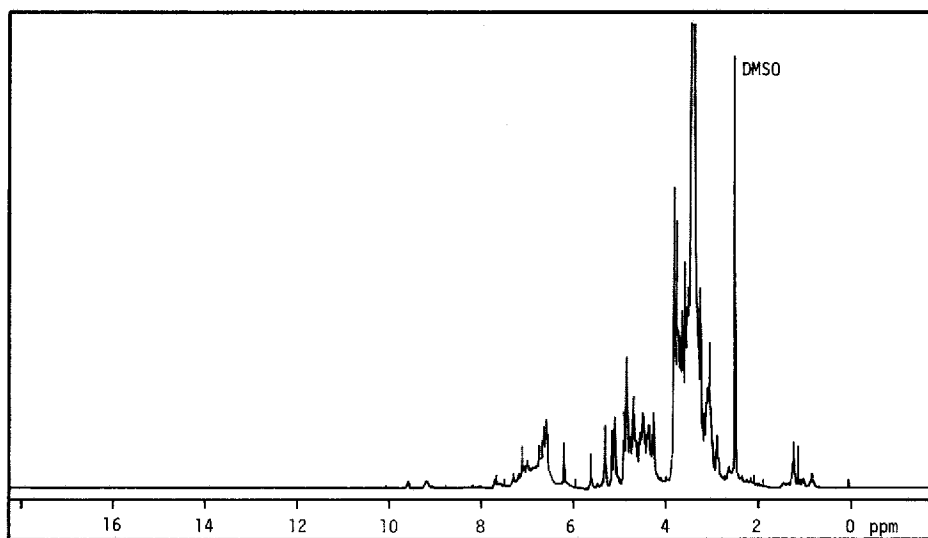
Fig. 6. ¹H NMR spectrum of the purified unknown pigment from cultured cells of safflower in [²H₆]dimethyl sulphoxide (DMSO) (300 MHz).
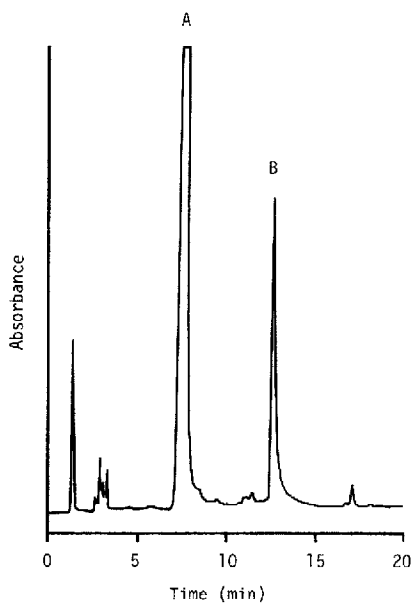

Fig. 7. Chromatogram of yellow pigments from petals of safflower. Column: Develosil ODS-5 (5 μm, 250 mm × 4.6 mm I.D.). Eluents: A, water; B, methanol; linear gradient from 10 to 60% B in 15 min; flow-rate, 1 ml/min. Detection: 410 nm (0.04 a.u.f.s.). Temperature: ambient. Injection volume: 10 μl. Peaks: A = safflor yellow A (or safflomin-A); B = precursor of carthamin.

Carthamin production by the reaction of a cultured cell homogenate and the precursor of carthamin

In order to observe the carthamin production, a few strips of filter-papers were added to a mixture of an homogenate of cultured safflower cells with either yellow peak A or B fractionated by RP-HPLC or Sep-Pak C_{18} cartridge. The filter-papers turned red only upon reaction with peak B. The red pigment produced was extracted with pyridine and analysed by RP-HPLC. Its retention time was identical with that of carthamin, as shown in Fig. 1a. Furthermore, the UV–VIS absorption spectrum in the visible region was the same as that of carthamin in Fig. 4a.

From these observations, it was concluded that the yellow pigment of peak B was due to the precursor of carthamin, and the cultured cells of safflower possessed the enzyme[5,6] catalysing the synthesis of carthamin from its precursor.

DISCUSSION

Based on the observation that the unknown pigment from cultured cells was immediately decomposed to light yellow matter in a solution of 1% methanolic hydrochloric acid, we inferred that this unknown pigment was not anthocyanin, one of the most typical of red flower pigments. Moreover, we discounted the possibility of carotenoids, because this unknown pigment was not soluble in benzene.

The unknown red pigment from cultured cells has several properties similar to those of carthamin. (a) Both pigments were adsorbed to cellulose, and extracted with pyridine strongly or acetone–methanol (or acetone–water) weakly from cellulose. These characteristics were utilized to extract both pigments. (b) Both pigments have high melting points (higher than 300°C) and so could not be analysed by conventional EI-MS. (c) Both pigments in water were very unstable at room temperature and in the presence of light, although when adsorbed to cellulose they were relatively stable. (d) Both pigments had similar R_F values in TLC on silica gel plates. (e) Both pigments were indicated to be glycosidic compounds by their IR, ^{1}H and ^{13}C NMR spectra.

On the other hand, both pigments showed several differences. (a) The unknown pigment from cultured cells had lower solubility in water than did carthamin. The $\lambda_{max.}$ of the former did not change significantly in aqueous solutions of different univalent and divalent salts, but the $\lambda_{max.}$ of the latter changed from 510 to 530 nm, indicating strongly the ionic structure of carthamin. (b) The retention time of the unknown pigment in RP-HPLC was longer than that of carthamin, as seen in Fig. 1, indicating the higher hydrophobicity of the unknown pigment. This property is in accord with the evidence in (a). (c) The UV absorption spectrum of carthamin showed a relatively broad main peak at around 522 nm and a broad peak at around 370 nm, but that of the unknown red pigment had a sub-peak at around 490 nm besides the main peak around 523 nm. The colour of freeze-dried carthamin was brick-red, but that of the unknown pigment was purple-blue. (d) Moreover, the IR, MS and ^{1}H NMR spectra of the pigments were remarkably different. (e) Both pigments were bleached in an alkaline solution of disodium carbonate. When neutral conditions were restored, carthamin became red again, but the unknown pigment remained bleached.

Based on the observations described above, it was concluded that the unknown red pigment produced in cultured cells of safflower differed from carthamin, antho-

cyanins and carotenoids, although it might be a glycosidic compound. Further experiments will be required to identify the unknown red pigment.

The enzyme which mediates the synthesis of carthamin from its precursor was found in the vegetative tissues or etiolated hypocotyl of safflower[5,6]. As preliminary experiments, we extracted the yellow pigment, the precursor of carthamin, from petals of safflower by RP-HPLC methods or a Sep-Pak C_{18} cartridge. We confirmed that the cultured cells of safflower possessed the enzyme activity, from the formation of carthamin upon reaction of an homogenate of cultured cells and the yellow precursor of carthamin. Therefore, it is assumed that the cultured cells lacked the biosynthesis pathway of the yellow precursor pigment.

REFERENCES

1 H. Obara and J. Onodera, *Chem. Lett.*, (1979) 201.
2 Y. Takahashi, N. Miyasaka, S. Tasaka, I. Miura, S. Urano, M. Ikura, K. Hikichi, T. Matsumoto and M. Wada, *Tetrahedron Lett.*, (1982) 5163.
3 C. Kuroda, *Nippon Kagaku Kaishi*, 51 (1930) 237.
4 T. R. Seschadri and R. S. Thakur, *Curr. Sci.*, 29 (1960) 54.
5 K. Saito, Y. Takahashi and M. Wada, *Z. Naturforsch., C.* 38 (1983) 724.
6 K. Saito, Y. Takahashi and M. Wada, *Biochim. Biophys. Acta*, 756 (1983) 217.
7 T. Murashige and F. Skoog, *Physiol. Plant.*, 15 (1962) 473.
8 H. Obara and J. Onodera, *Chem. Lett.*, (1978) 643.
9 J. Onodera, H. Obara, M. Osone, Y. Maruyama and S. Sato, *Chem. Lett.*, (1981) 433.