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Purification and some characteristics of precarthamin, a precursor of carthamin, isolated from the florets of *Carthamus tinctorius* L.

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Abstract

An unstable flame-coloured pigment, tentatively named precarthamin, was isolated as orange-yellow microneedles with 0.12% yield from the yellow florets of *Carthamus tinctorius* L. through many purifying steps. The precarthamin could be enzymically converted to a red pigment, which was identified as carthamin by its chromatographic and spectral properties. The content of precarthamin was examined at the various growth stages of the *Carthamus* flowers.

Key words: Carthamus tinctorius L., safflower, precarthamin, carthamin.

INTRODUCTION

The dried florets of safflower (*Carthamus tinctorius* L.), are generally used as a plant source for a red dyestuff or for a vasodilator utilized in medecine. The pigment in the flower has been studied by various workers (Mayer and Cook 1943). Kuroda (1930) proposed structure (Fig. 1, I) for the pigment, carthamin, which was extracted from commercial carthamin paste imported from China, and the same author showed that carthamin gave a yellow unstable tautomeric form of isocarthamin (Fig. 1, II) by dilute hydrochloric acid treatment.

In later studies, a tentative formula (Fig. 1, III) had been proposed by Obara and Onodera (1979) for carthamin. We have also rendered a new formula (Fig. 1. IV) combining two equatorially C-1-alkylated glucopyranosides into the bichalcone skeleton (Takahashi et al. 1982).

Carthamus flowers in full bloom are yellow in colour, but the tinge changes to orange and finally red. Wada (1953) proved that a precursor of carthamin obtained from fresh yellow florets could be turned to a red

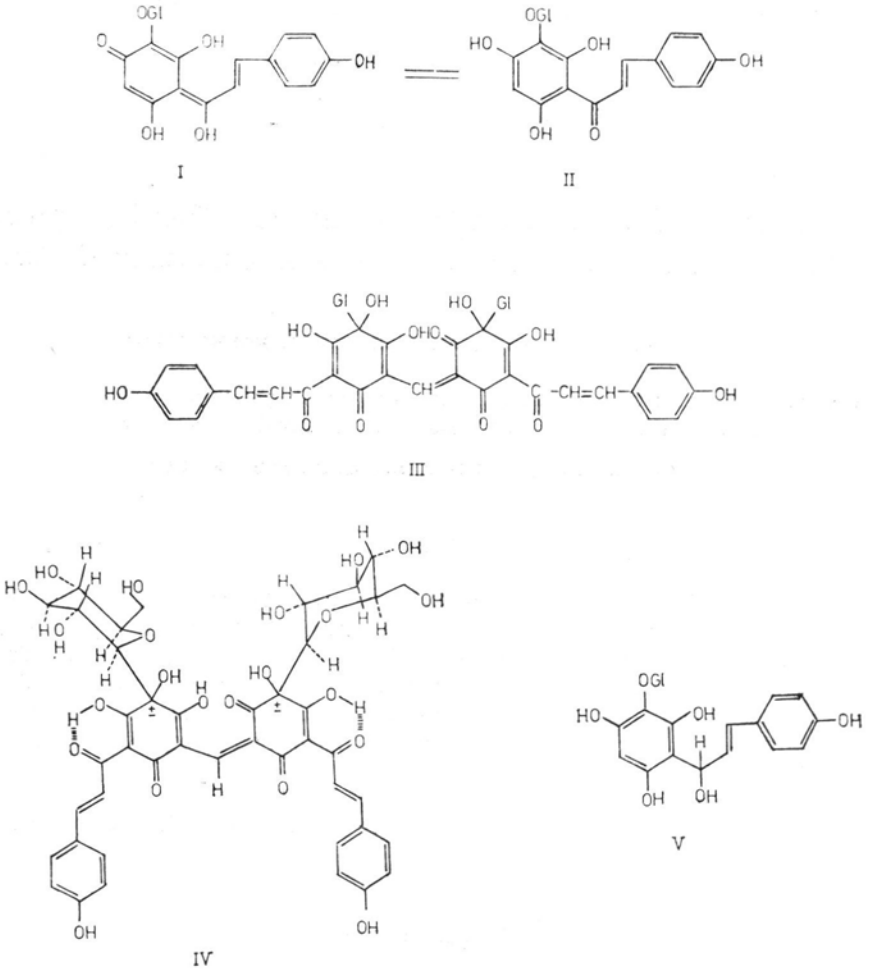

Fig. 1. Structures of carthamin and precursor of carthamin. I — carthamin (by Kuroda 1930), II — isocarthamin (by Kuroda 1930), III — carthamin (by Obara and Onodera 1979), IV — carthamin (by Wada et al. 1982), V — precursor of carthamin (by Shimokoriyama and Hattori 1955)

pigment, presumably carthamin, by extracts of young *Carthamus* flowers. Shimokoriyama and Hattori (1955) reported a tentative structure (Fig. 1, V) for the precursor of carthamin on the basis of a study with crude ethanol powder of *Carthamus* flowers.

In our previous paper, we reported that carthamin was synthesized from a precursor by a cell-free extract of mature flowers (Saito et al. 1983a). However, the precursor used in the previous work was not pure enough to be crystallized. We report here that precarthamin was obtained in crystalline state from fresh yellow florets of *C. tinctorius* and that it could be biologically converted into carthamin. The catabolic process of

precarthamin is also briefly discussed relating to changes in the content of the pigment in florets at various growth stages of the flowering plant.

MATERIAL

Samples of *Carthamus* flowers were collected at our experimental field in July, 1977 and/or a farm of Yomeishu, Co. Ltd., Kushikida, Kagoshima, Japan, in June, 1982. The picked yellow florets (2.09 kg) were finely blended in methanol with a homogeniser and washed with ether, followed by vacuum drying to a constant weight (235 g). Another sample of *Carthamus* flower for quantitative studies of precarthamin was provided from our experimental field in July 1982. The yellow flower florets without ovaries were classified into the following groups: group one 1.00-1.31 cm (899 mg), group two 1.31-1.70 cm (1214 mg), group three 1.71-2.10 cm (1318 mg), group four 2.11-2.50 cm (1351 mg), group five 2.51-2.90 cm (1339 mg), group six 2.91-3.30 cm (1376 mg).

METHODS

COLUMN AND THIN-LAYER CHROMATOGRAPHY

All chromatographic solvents used were of spectral grade. For column chromatography, Avicel cellulose (Asahikasei, Co. Ltd., Osaka, Japan) and Toyo Pearl (HW-40F and HW-40SF, Toyo Soda, Co. Ltd., Tokyo, Japan) were used exclusively. Column chromatography was carried out under argon atmosphere. Thin-layer chromotography (TLC) was accomplished on cellulose or silica gel G (E. Merck), and the compounds were detected in UV light (254 nm) and/or by spraying $FeCl_3$ in ethanol, which gave a dark green colour, or by spraying $KMnO_4$ in acetone, which gave a red colour. The chromatograms were developed in the following solvent systems (by vol.): (A) 1-butanol:acetic acid:water — 4:1:2; (B) 1-butanol:acetic acid: water — 4:1:5 (upper layer); (C) 1-butanol: :ethyl acetate:methanol:water — 4:4:1:2; (D) 1-butanol:ethyl acetate: :methanol:water — 4:9:1:2; (E) methanol:acetic acid: water — 6:1:4; (F) 1-butanol:ethyl methyl ketone:pyridine:water — 8:3:1:2; (G) phenol: :acetic acid:water — 40:1:10; (H) methanol:water — 65:35 and (I) acetone: :water — 40:60.

SPECTRAL ANALYSES

Infrared spectra were recorded on a Shimadzu Model IR-400 spectrophotometer, using potassium bromide disks of the pigment. ^1H NMR spectra were obtained with a Nihondenshi FX-400 spectrometer in Pyridine-d_5/Methanol-d_4. Ultraviolet spectra were registered on a Shimadzu MPS-2000 spectrophotometer.

ISOLATION AND PURIFICATION OF PRECARTHAMIN FROM FLORETS

In a preliminary experiment, 5 g of dried and finely powdered florets were extracted exhaustively with 30% (v/v) formic acid in methanol (6 times). The resulting extracts were concentrated to a small volume with a rotary evaporator and the brownish-yellow concentrate (1.48 g) was passed through a column of Amberlite IR-120B (H^+-form, 2×30 cm). The transit liquor was condensed *in vacuo* and the residue (700 mg) was separated twice on the cellulose column with solvent-(A), and then twice again on the column with solvent-(C). Precarthamin fractions were evaporated to dryness and the mass (61.7 mg) was purified on a Toyo Pearl (HW-40F) column (1.7×100 cm) with solvent-(E). Orange-yellow pigment (12 mg) was obtained at the final purification step.

In large scale experiments, isolation and purification of precarthamin were done as follows. Dried and finely powdered florets (230 g) were extracted exhaustively with 30% (v/v) formic acid in methanol, each time for 10 min at room temperature. The combined extracts (28 dm^3) were evaporated *in vacuo* at below 30°C to give a dark-brown gummy residue (48.4 g). The residue was dissolved in water, passed through a column of Amberlite IR-120B (H^+-from, 500 cm^3, column size: 4×80 cm) and washed with water. The resulting washings (6 dm^3) were freeze-dried *in vacuo*. The brown powder (27.7 g) was dissolved in 120 cm^3 of solvent-(A) and loaded on a column (7×150 cm) packed with 2.4 kg of Avicel cellulose which had been previously washed with 10 dm^3 of the same solvent. Fractions (15 g each) were collected, and each fraction was checked on a cellulose TLC plate with solvent-(A). Precarthamin was distinguishable from other pigments by its R_f values on chromatograms and the colour visualised by spraying $KMnO_4$ in acetone. The precarthamin-containing fractions (No. 7-47) were evaporated *in vacuo* at room temperature to give a reddish-brown powder (10 g). The crude powder was chromatographed on a column (5×120 cm) with 700 g of cellulose by using solvent-(D). Fractions (No. 61-162) showing a single spot with R_f value 0.51 were combined. Removal of the solvent gave orange-yellow precarthamin (1.09 g). The pigment was parified on a column of Toyo Pearl (HW-40F) equilibration with solvent-(H) (column size: 4×70 cm, gel bed: 3.5×55 cm; 530 cm^3, flow rate: 25 $cm^3 \cdot h^{-1}$, fraction: 7 g). Evaporation of the fractions (No. 203-241) gave an orange-yellow crystalline solid (330 mg). The crystals were further purified under the same conditions (column size: 3×70 cm, gel bed: 2.6×52 cm; 280 cm^3, flow rate: 23 $cm^3 \cdot h^{-1}$, fraction: 5 g). Flame-coloured microneedles (265 mg, yield 0.12% based on dried florets mass) were obtained in the final purification step. Recrystallization of the crystals from aqueous methanol (long

standing) gave fine microneedles of precarthamin. It showed slight discolouration at about 150°C and then slowly blackened with rising temperature.

SEPARATION OF CARTHAMIN SYNTHESIZED BY ENZYME REACTION

Ten mg of precarthamin in 50 cm^3 of citrate buffer (50 mM, pH 5.2) were mixed with 5 g of cellulose and 2 cm^3 of enzyme extract which was prepared from young safflower seedlings (S a i t o et al. 1983b) and incubated in an incubator (96 strokes $\cdot h^{-1}$) at 30°C for 17 h. The carthamin adsorbed cellulose was separated from the reaction mixture by centrifugation, and then washed four times water (80 cm^3). The well-washed reddish cellulose was extracted five times with 60% (v/v) acetone (120 cm^3), and the combined acetone extracts were evaporated *in vacuo* at below 30°C to give a red mass (21 mg). Purification of the mass was carried out on a column of Toyo Pearl (HW-40F) with solvent-(I) (column size: 20×60 cm, gel bed: 1.8×36 cm; 92 cm^3, flow rate: 23 $cm^3 \cdot h^{-1}$, fraction: 5 g). The chromatographically pure reddish mass (4.9 mg) was obtained from the eluate containing carthamin after removal of the solvent under reduced pressure at less than 30°C.

PREPARATION OF AN AUTHENTIC CARTHAMIN

Florets were treated as follows: crushed in air, pressed out and dried in shade. The dried florets, which were washed several times with acetone and water were extracted with 0.5% (w/v) K_2CO_3 and then filtered by suction. White muslin was immersed in the combined extracts and acidified with 10% (w/v) citric acid. Carthamin-adsorbed muslin was washed well with water and exhaustively extracted with 60% (v/v) acetone. The extracts were evaporated *in vacuo* at below 30°C to gave a greenish-red mass with 0.35% yield from dried florets. The crude carthamin was recrystallized several times in 60% (v/v) acetone to give red needles with 0.007% yield converted to fresh florets.

STUDY ON PRECARTHAMIN CONTENT IN THE FLORETS AT VARIOUS GROWTH STAGES

The freshly collected yellow florets were crushed in water (8 cm^3) and allowed to stand for 2 days at room temperature. Reddish florets were filtered by suction and then washed with 100 cm^3 of water. The washed reddish florets were extracted with 100 cm^3 of 0.5% (w/v) K_2CO_3. Twenty five cm^3 of the combined extracts were made up to 100 cm^3 with 0.5% (w/v) K_2CO_3 and absorbance at 365 nm was measured. The precarthamin content was calculated from a standard curve.

RESULTS

CHARACTERISTICS OF PRECARTHAMIN

Dried and finely powdered florets of *Carthamus tinctorius* were thoroughly extracted with formic acid in methanol at room temperature until the extract gave negative spot against the potassium permanganate reagent which was used for detecting precarthamin. The precarthamin in the extracts could be detected along with many other yellowish pigments by either analytical liquid chromatography (Toyo Pearl HW--40SF) or cellulose TLC. The isolation and purification of the pigment were achieved by cellulose column chromatographic techniques as described in the experimental section. Purified precarthamin is a flame-coloured pigment which has UV absorption peak at 225 (sh.), 243, 340 (sh.) and 423 nm (Fig. 2). This compound is soluble in water, methanol, ethanol and pyridine.

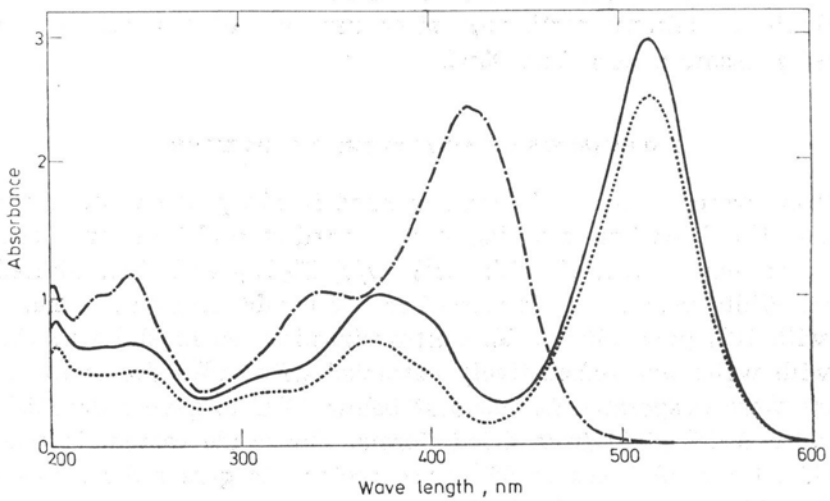

Fig. 2. UV spectra of enzymically synthesized product, authentic carthamin and precarthamin in methanol. ——— authentic carthamin (0.05 mg·cm^{-3}); - - - - synthesized product (0.09 mg·cm^{-3}); — - — precarthamin (0.035 mg·cm^{-3})

Table 1 shows R_f values and colour reactions of the purified compound. The precarthamin gave a greenish-yellow colour with ethanolic ferric chloride and showed a negative reaction with 8% (w/v) sodium hydroxide (see Table 1). Precarthamin in solid state was liable to change from orange-yellow to reddish-yellow within several weeks or overnight on cellulose TLC plates. However, stabilization of the pigment could be achieved by storage at —20°C in an atmosphere of argon gas for at least 6 months without appreciable change in colouration.

Table 1

R_f values and colour reaction of precarthamin

Solvent	R_f value	Colour reaction				
		8% NaOH	Mg—HCl	HCl	H_2SO_4	$FeCl_3^a$
A	0.63	yellow	yellow--brown	yellow	yellow	greenish--yellow
B	0.64					
C	0.46					
D	0.23					

A: 1-butanol/acetic acid/water (4: 1: 2, by vol.), B: 1-butanol/acetic acid/water (4: 1: 5, by vol., upper layer), C: 1-butanol/ethyl acetate/methanol/water (4: 4: 1: 2, by vol.), D: 1-butanol/ethyl acetate/methanol/water (4: 9: 1: 2, by vol),

[a] ethanol solution

IDENTIFICATION OF THE ENZYMICALLY SYNTHESIZED PRODUCT

Table 2 presents the R_f values of the enzymically synthesized product and an authentic carthamin on silica gel TLC plates. The values of both red pigments agreed reasonably well with each other.

Table 2

R_f values of enzymically synthesized product and authentic carthamin on silica gel plates in three solvents

Solvent	R_f value	
	a	e
B	0.43	0.44
F	0.70	0.70
G	0.30	0.28

B: 1-butanol/acetic acid/water (4: 1: 5, by vol., upper layer), F: 1-butanol/ethyl methyl ketone/pyridine/water (8: 3: 1: 2, by vol.), G: phenol/acetic acid/water (40: 1: 10, by vol.), a: authentic carthamin, e: enzymically synthesized product

Each UV spectrum of the two red pigments has proved to be identical (Fig. 2). Furthermore, comparison of IR spectra between the enzymic product and an authentic carthamin displays good similarity with each other in their spectral properties (Table 3).

Figures 3 and 4 show NMR spectra of authentic carthamin and the enzymically synthesized product measured at 400 MHz in Pyridine-d_5 /Methanol-d_4. The two spectra obviously reflect the same chemical structure of the two pigments, though small discrepancies are seen at proton 4″ and 6″ (4.03 t and 3.99 bt, 4.75 d and 4.65 bd, 4.26 dd and 4.24 bdd each). These slight differences may result from low purity of the enzymically synthesized product.

Table 3

IR spectra data of enzymically synthesized product and authentic carthamin

Pigment	Wave length, cm^{-1}				
a	3400(s)	2920(w)	1620(w)	1600(w)	1500(s)
	1410(m)	1355(m)	1260(s)	1165(s)	1080(w)
	1030(s)	950(m)	825(w)	705(w)	—
e	3400(s)	2920(w)	1620(w)	1600(w)	1510(s)
	1406(w)	1358(w)	1260(s)	1165(s)	1075(w)
	1030(m)	950(w)	830(w)	710(w)	—

a: authentic carthamin, e: enzymically synthesized product, s: strong, m: medium, w: weak

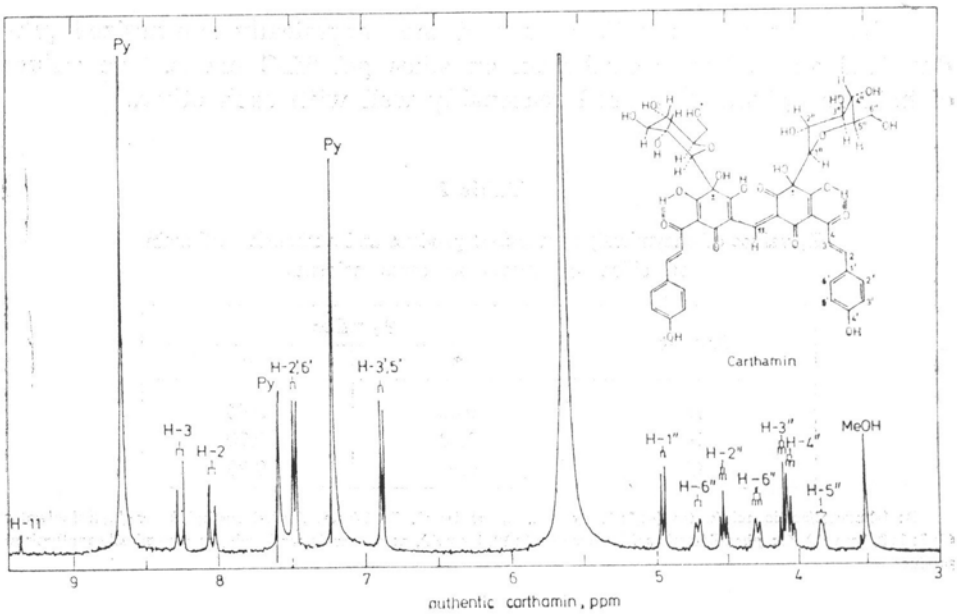

Fig. 3. ^1H NMR spectrum of authentic carthamin recorded at 400 MHz in Pyridine-$-d_5$/Methanol-d_4 (95:5, by vol.)

QUANTITATIVE RELATION OF PRECARTHAMIN IN THE FLORETS

The determination of precarthamin was carried out by an indirect method as described in the experimental section. Figure 5 shows the content of precarthamin in florets and the fresh weight of the collected florets at six growth stages. The precarthamin content decreases with the maturation of florets.

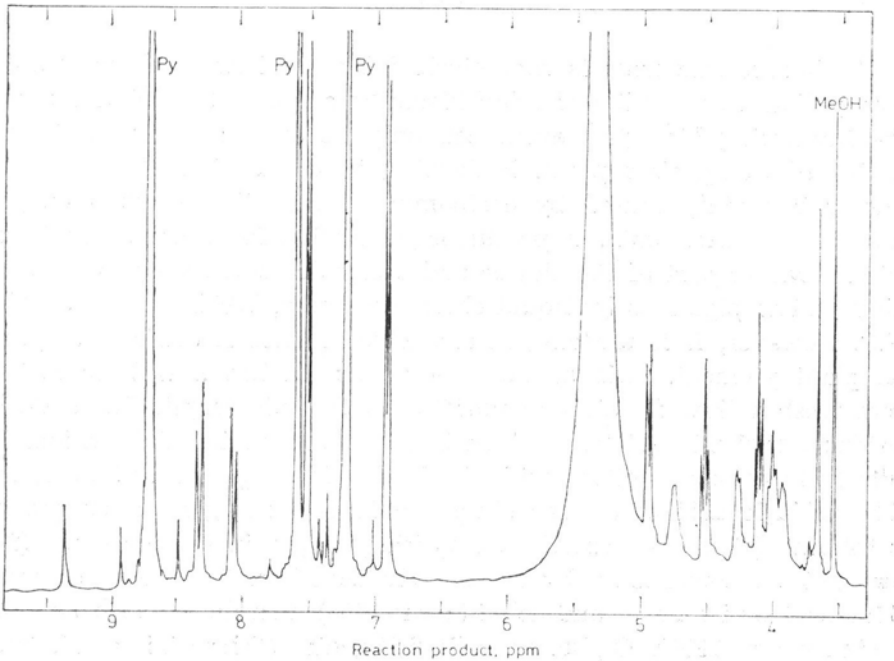

Fig. 4. ^{1}H NMR spectrum of enzymically synthesized product recorded at 400 MHz in Pyridine-d_5/Methanol-d_4 (95:5, by vol.)

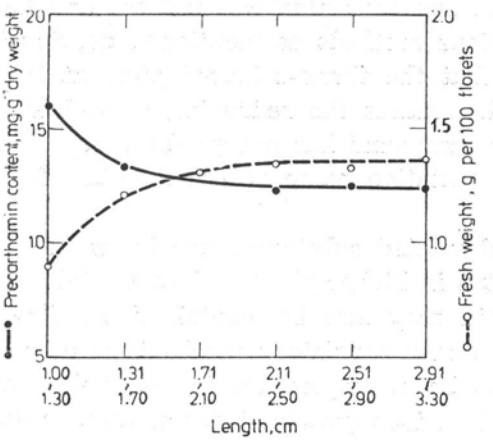

Fig. 5. Change in precarthamin content of tubular flowers from *C. tinctorius* L. Uniform tubular flowers without ovaries were divided into six groups as illustrated in the figure. Fresh weight of each sample was determined by using a portion of the uniform flowers

DISCUSSION

Carthamus tinctorius is characterized by the flower colour changing from yellow to red after the full-blooming stage of the tubular flower. The interesting biological event can only be seen specifically in this species of *Compositae* plant. Reddening in colouration of the yellow flowers is mainly caused by carthamin accumulation through enzymic processes. Precarthamin, a precursor of carthamin is contained in the yellow tubular part of the flower and easily detectable along with many other yellow pigments by liquid chromatography, UV light and cellulose TLC. However, it is unstable in air, which makes it difficult to purify the plant pigment. This unstable precarthamin has now been isolated from fresh yellow florets and purified to the microcrystalline state *via* various purification steps as described in the experimental section. Results from colour reactions with ferric chloride, aqueous sodium hydroxide, sulfuric acid and other chromogenic reagents give us suggestions as follows: (1) it may contain o-dihydric group(s) in the molecule, (2) it may not be constructed by normal chalcone structure, e.g., coreopsin (Shimokoriyama and Hattori 1953), marein (Harbone and Geissman 1956), C-glucosylisoliquiritigenin (Ohashi et al. 1977). The UV absorption pattern also indicates that the compound may be a novel type of colouring matter that has not yet been isolated from any other flowering plant. Analytical studies on the structure of precarthamin are now undertaken in our laboratories.

It is interesting to know if the precarthamin can really be converted *in vitro* to carthamin. We tested enzymic synthesis of carthamin by using the purified pigment as an enzyme substrate. A red pigment resulting from the enzymic reaction was isolated and identified as carthamin by using various methods as mentioned previously. The above results confirm us that the flame-coloured pigment is the real precursor of carthamin which causes the reddening of yellow tubular flowers of *C. tinctorius*. This compound has not yet been reported in literature and we have given a tentative name "precarthamin" for the novel plant pigment.

Flavonoids and related substances are known to exist in a state of dynamic equilibrium in higher plants (Zenk 1967, Barz and Hösel 1975). Precarthamin may also be contained in flower tissues at an equilibrated state under regulatory mechanisms of enzyme action. Therefore, it seems to be of importance to study the quantitative relation of precarthamin at various growth stages of tubular flowers. We selected six different stages for the flower petals and examined very closely the content of precarthamin in each batch as illustrated in Fig. 4. The fresh weight of the flower increases slowly up to 2.1-2.5 cm by length, while, on the contrary, the content of precarthamin decreases gradually at that

stage. This indicates the fact that the pigment is actively used within flower tissues, which reflects the depression of the content during the growing stage of the florets. Reddening of yellow petals occurs at the later phase of the flowering period, where almost all tubular flowers reach more than about 3.5 cm in length. The flower petals at that stage are readily changed from yellow to dark red on the flower disks. Precarthamin may be enzymically catabolised to carthamin as well as other related substances prior to the appearance of the atractive colour transit reaction. These observations present interesting problems from the metabolic or enzymological point of view.

Acknowledgments

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Oczyszczanie i charakterystyka prekartaminy, prekursora kartaminy, wydzielonej z kwiatów Carthamus tinctorius L.

Streszczenie

Wydzielono prekartaminę, prekursor kartaminy w postaci pomarańczowożółtych igieł ze świeżych kwiatów języczkowych *Carthamus tinctorius*. Przekształcono prekartaminę w czerwony barwnik (kartaminę) *in vitro*, stosując preparat enzymatyczny. Dane uzyskane po rozdziale chromatograficznym z analizy spektralnej, UV, IR i NMR były zgodne z odpowiednimi danymi dla kartaminy wyodrębnionej z kwiatów. Zawartość prekartaminy w kwiatach języczkowych oznaczono kolorymetrycznie w różnych stadiach wzrostu kwiatów. Uzyskane dane wskazują, że ilość tego barwnika w kwiatach maleje wraz z ich rozwojem.