Distribution and Variation of the Catalytic Activity of the Enzyme Responsible for Carthamin Synthesis in Safflower Seedlings

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Catalytic activity of the enzyme which mediates the synthesis of carthamin was examined in the etiolated hypocotyl tissues of *Carthamus tinctorius* L. The enzyme was optimally active at pH 5.2 in citrate buffer. The activity of the enzyme was highest in the extract from 2-day-old seedlings after sowing and it declined sharply with tissue ageing. The enzyme activity was localized in the apical region of the seedlings and moreover it was preferentially associated with the cytosol fraction.

Introduction

Carthamin, 6-β-D-glucopyranosyl-2-[[3-β-D-glucopyranosyl-2,3,4-trihydroxy-5-[3-(4-hydroxyphenyl)-1-oxo-2-propenyl]-6-oxo-1,4-cyclohexadien-1-yl]methylene]-5,6-dihydroxy-4-[3-(4-hydroxyphenyl)-1-oxo-2-propenyl]-4-cyclohexene-1,3-dione (Scheme I), arises in flower petals and causes in red pigmentation of the florets of *Carthamus tinctorius* L. In a preceding study [1] we observed that a cell-free preparation from this plant at the full blooming stage could synthesize carthamin from a putative precursor under aerobic conditions. The enzyme activity was detected widely in above-ground parts of safflower and moreover the highest activity of the enzyme was found to be localized in younger and developing tissues, though carthamin appears only limited part of the mature petals.
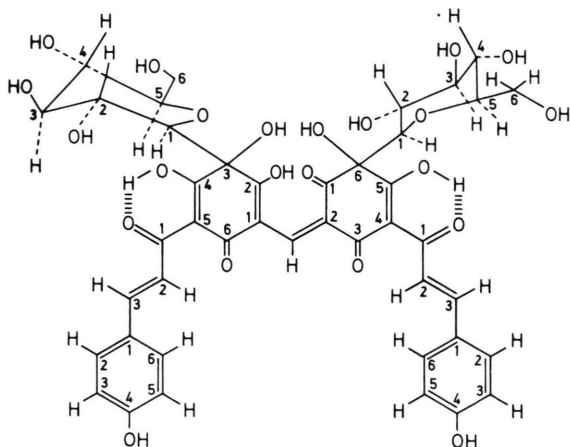
It seems to be interesting to know whether or not carthamin-synthesizing enzyme is also operative in younger seedlings.

This paper deals with evidence from studies on distribution and variation of the catalytic activity of the enzyme in etiolated hypocotyls of safflower seedlings.

Scheme I. Structural formula of carthamin.

Materials and Methods

Materials

Seeds of *C. tinctorius* were obtained locally. Soaked seeds (semi-sterilized) were germinated in trays with moist vermiculite in the dark at 27 °C. After given times of germination period, the etiolated hypocotyls were cut off from the roots and the excised hypocotyls were used as the source of enzyme preparation. The chemicals used in this study were obtained from the following sources: Sephadex G-25, Pharmacia Fine Chemicals (Uppsala, Sweden); Toyo Pearl HW-40F (Polyvinyl type adsorption packings), Toyo Soda Co., Ltd. (Tokyo, Japan); Silica gel thin-layer plates, Merck (Darmstadt, West Germany); Sodium D-araboascorbate, Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan); Dithiothreitol, Nakarai Kagakuyakuhin Kogyo Co., Ltd. (Kyoto, Japan). All other chemicals used in this study were of an analytical grade of purity.

Preparation of a precursor of carthamin

Finely blended and dried powder of *Carthamus* flowers was extracted 6 times, stirring with a magnetic stirrer in rectified methanol containing 30% (v/v) formic acid, each time for 10 min at room temperature. Pooled filtrates were evaporated using a rotary evaporator at less than 30 °C. The brownish yellow powder (412 mg) was dissolved in 3–4 ml of distilled water and passed through a column of Amberlite IR-120B (H^+-form, 1.5×24 cm). The resulting transit liquor (60 ml) was freeze-dried with liquid nitrogen under reduced pressure and stored in a freezer at −20 °C. Brownish yellow powder (255 mg) was dissolved into *n*-butanol/acetic acid/water (4:1:2, v/v/v) and loaded onto a cellulose column (2.0×50 cm). Chromatography was done using the same solvent with continuous bubbling of argon gas. Yellowish eluents were condensed with an evaporator and resulting concentrates were rechromatographed under the same condition as described above. Eluted fractions were examined by spot test using a spray reagent of 100 mM potassium permanganate in 85% (v/v) acetone on thin-layer plates and precursor-containing fractions were concentrated *in vacuo* at less than 30 °C, followed by freeze-drying under reduced pressure. Brownish yellow powder obtained (48 mg) was dissolved in a minimum amount of 65% (v/v) methanol and chromatographed on a column of Toyo Pearl HW-40F (2.0×25 cm). Each 10-fraction was checked at intervals on cellulose thin-layer plates by spraying potassium permanganate reagent. The carthamin precursor containing fractions were collected and evaporated to dryness *in vacuo*. Dark yellow powder obtained (5.8 mg) was stored at −20 °C as the enzyme substrate in a small vessel, whose air was replaced with argon gas.

Preparation of enzyme extracts

All the operations were carried out in the cold. A known weight of etiolated hypocotyls was washed three times in distilled water and dipped in cold 50.0 mM citrate-phosphate buffer, pH 7.0, containing 20.0 mM D-araboascorbate, at the rate of 2–3 ml of buffer per g fresh weight of hypocotyls. The hypocotyls were homogenized in a pre-chilled Waring blendor for 10 s and filtered through cheese-cloth. The extract was centrifuged for 10 min at $15000 \times g$ in a refrigerated centrifuge. The pellet

was discarded and the clear supernatant was brought to 30% saturation with solid ammonium sulfate. After standing for 10 min with continuous stirring with a magnetic stirrer in the cold at 2 °C, the precipitate was collected by centrifugation and discarded. The supernatant was brought to 80% saturation with ammonium sulfate and the resulting precipitate was collected by centrifugation. The pellet was then dissolved in about one-third of the original volume of 50.0 mM citrate-phosphate buffer, pH 7.0 and passed through a column of Sephadex G-25 (0.9×30 cm). Elution was carried out with the same buffer and the protein fractions were used for enzyme assay.

Cellular fractionation

Etiolated hypocotyls excised from 3-day-old seedlings (Parts I and II, 32.8 g fresh wt. see Fig. 5 in Results and Discussion) were homogenized for 10 s in two vol. per g fresh weight of cold 50.0 mM citrate-phosphate buffer, pH 7.0 containing 250.0 mM mannitol, 5.0 mM $MgCl_2$, 5.0 mM KCl, and 1.0 mM dithiothreitol. After initial centrifugation at $170 \times g$ for 5 min to remove cell debris, some particulate fractions were separated by differential centrifugation at $600 \times g$ for 5 min, $12000 \times g$ for 30 min, and $100000 \times g$ for 60 min. Each of the centrifuge pellets was suspended in 50.0 mM citrate-phosphate buffer, pH 7.0 and retained in the cold at 2 °C for the enzyme assay.

Assay of enzyme activity

The enzyme activity was assayed spectrophotometrically by measuring initial changes in absorbance at 517 nm during the incubation period. The assay medium composed of enzyme extract (0.12–1.38 mg protein), 0.5–1.0 µg substrate and 50.0 mM citrate buffer, pH 5.2 in a total volume of 6.0–6.5 ml. The assay mixture was incubated for 10 min in open test-tubes at room temperature (26–28 °C). Carthamin content was computed from a calibration curve. The amount of protein was estimated using the folin phenol reagent by the method of Lowry *et al.* [2].

Extraction and purification of enzymically synthesized carthamin

Two grams of finely blended powder of safflower petals were boiled in methanol for 10 min. The

denatured powder was dried in a vacuum desiccator over silica gel for about 70 h and incubated with an enzyme preparation (75.5 mg protein) in 50 ml of 50.0 mM citrate buffer, pH 5.2 in an incubator (Yamato, Model BT-46) for 15 h at 30 °C with gentle shaking (90–102 strokes/h). The enzyme reaction was stopped by addition of methanol in the incubation mixture. The mixture was then centrifuged for 10 min at $1200 \times g$ and the supernatant was discarded. A resuspension of the precipitate was centrifuged separately four times and the cleanly washed precipitate was extracted with 25 ml of 0.5% K_2CO_3. The suspension was centrifuged for 3 min at $1600 \times g$ and the supernatant was pooled in the cold at 2 °C. The extraction procedure was repeated further six times and the pooled extracts (175 ml) were acidified with 20 ml of 10% citric acid. Four grams of cellulose powder were mixed into the acidic extract and stirred gently with a magnetic stirrer for about 1 h. Carthamin adsorbed powder was washed with distilled water until yellow impurities were washed out, and then exhaustively extracted with 60% (v/v) acetone. The combined extracts (160 ml) were concentrated at below 30 °C. Dark reddish mass (9.4 mg) was dissolved in a small volume of 40% (v/v) acetone and passed through a column of Toyo Pearl HW-40F (2.0×40 cm) which had been equilibrated previously with the same solvent. The acetone eluent (200 ml) was evaporated at less than 30 °C to give a dark reddish mass (1.6 mg).

Identification of the reaction product

The reaction product was identified by the colour, R_f values, and spectra inclusive shifts, often by comparison with an authentic sample. The R_f values were examined on silica gel thin-layer plates using *n*-butanol/acetic acid/water (4:1:2, v/v/v), *n*-butanol/pyridine/water (10:3:1, v/v/v), and ethyl-acetate/pyridine/water (14:5:3, v/v/v) as the developing solvent systems. UV-spectra were measured with a spectrophotometer (Shimadzu, Type 200). IR-spectra were recorded in KBr pellets using a Shimadzu spectrophotometer (Model IR-400).

Results and Discussion

When the etiolated hypocotyls of safflower seedlings were crushed with a pestle and mortar in a

Table I. Comparison of R_f values between enzymically synthesized product and an authentic carthamin.

Solvent system[a]	Enzymically synthesized product	Authentic carthamin
A	0.45	0.46
B	0.71	0.70
C	0.57	0.57

[a] A: *n*-butanol/acetic acid/water (4:1:2, v/v/v);
  B: *n*-butanol/pyridine/water (10:3:1, v/v/v);
  C: ethyl-acetate/pyridine/water (14:5:3, v/v/v).
Each solvent was developed ten centimetres on silica gel plates (3×12 cm) at room temperature (19–21 °C).

buffer solution containing carthamin precursor, the homogenate turned slowly to reddish orange and then to dark red, suggesting the possibility that the enzyme responsible for carthamin synthesis may operate also in the seedling tissues. This preliminary observation led us to investigate the enzymic synthesis of carthamin with extracts from etiolated hypocotyls of safflower seedlings. The enzymically synthesized carthamin was identified by colour, R_f values and spectroscopic properties, comparing with those of an authentic sample. The R_f values of the reaction product in three solvent systems are presented in Table I. The values are almost the same as those of an authentic carthamin which was obtained according to the method ref. [3]. UV-spectra of the incubation product were compared with those of an authentic specimen. Fig. 1 proves that two UV absorption spectra, E (enzymically synthesized product) and A (authentic carthamin) are essentially coincident with each other. Confirmatory evidence for the identity of the reaction product was also obtained by examining its IR absorption spectrum. The spectrum of the enzymically synthesized sample is superimposable with that of the standard carthamin (Fig. 2). As shown in Fig. 3a, catalytic activity of the enzyme in 50.0 mM citrate buffer had a maximum at pH 5.2 and a shoulder between pH 3.5 and 4.5. A similar value of optimum pH was also seen in 50.0 mM phosphate buffer, though less reactivity of the enzyme and no shoulder was observed in this buffer (Fig. 3b). Under the condition of the present experiment, non-enzymic conversion of carthamin precursor to carthamin could not be observed at the pH range used, when reaction was carried out with no enzyme extract or with boiled one in the incubation
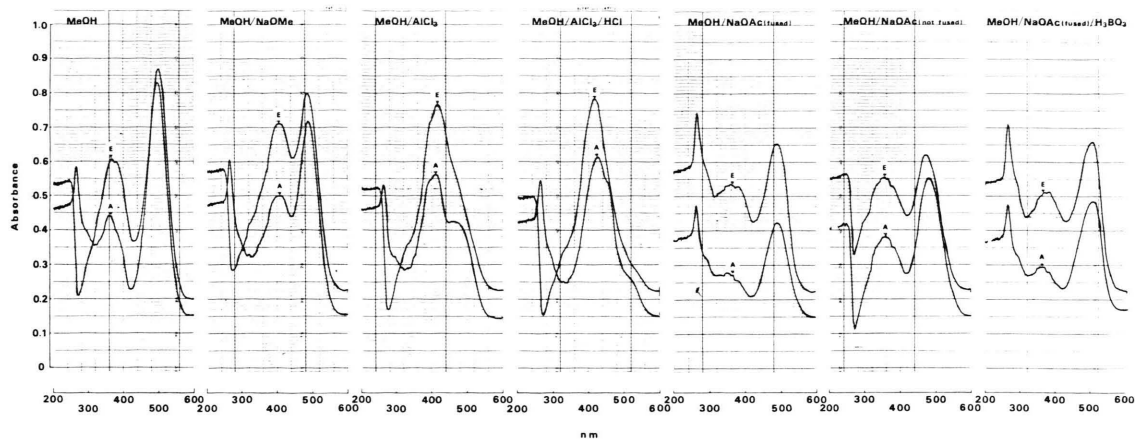
Fig. 1. Comparison of spectrophotometric properties between enzymically synthesized product and an authentic carthamin. E: enzymically synthesized product, A: authentic carthamin.
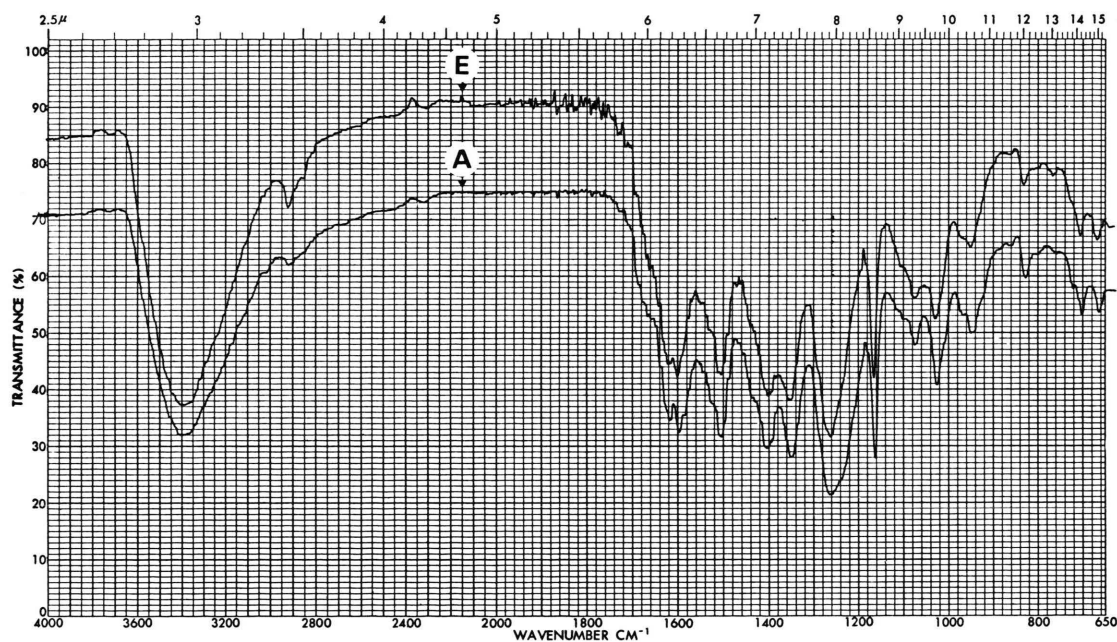

Fig. 2. IR spectra in potassium bromide pellets of the enzymically synthesized product and an authentic carthamin. E: enzymically synthesized product, A: authentic carthamin.

medium. Results of the enzyme preparations obtained from different aged seedlings clearly indicate that there is a marked peak of the activity during ageing of the etiolated seedlings (Fig. 4); the highest activity was detected in the extract from 2-day-old hypocotyls after sowing and decreased rapidly after 3 days. Fig. 5 shows the enzyme activity in various parts of the etiolated hypocotyls excised from 3-day-old seedlings. The activity varied in different parts examined. The highest value was found to be located in the apical area and then the enzyme activity decreased gradually toward the basal region. Subcellular location of the enzyme was examined using a differential centrifugation technique, revealing that the enzyme activity was localized mainly in the soluble
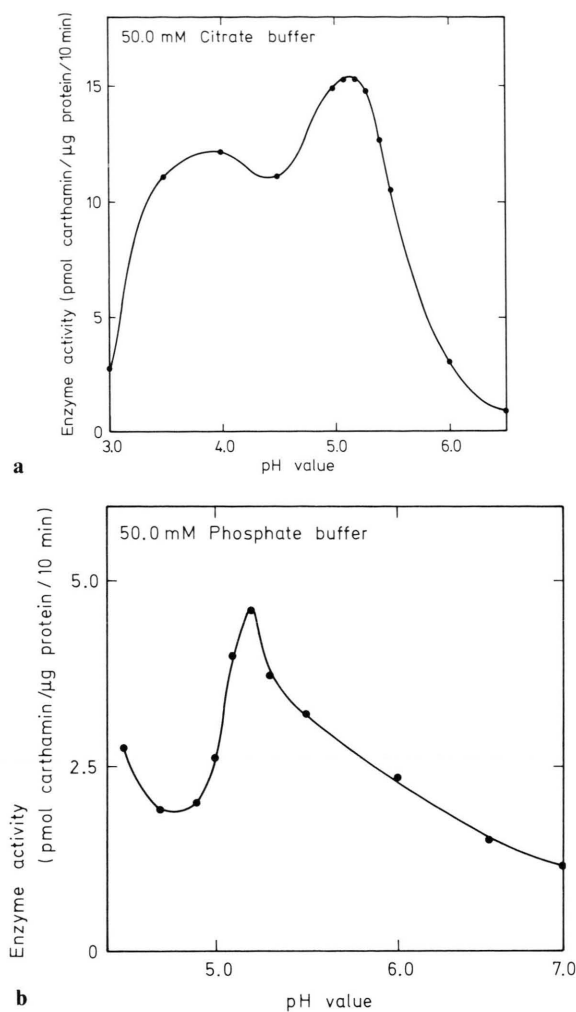
a

b

Fig. 3. Effect of pH value on the enzymic synthesis of carthamin. (a) Results from tests in 50.0 mM citrate buffer and (b) in 50.0 mM phosphate buffer. 4-Day-old seedlings (22.6 g fresh wt) were used for enzyme preparation. For details of enzyme preparation and enzyme assay see Materials and Methods.

Table II. Subcellular distribution of the enzyme responsible for carthamin synthesis.

Fraction	Enzyme activity [μg carthamin/10 min]	Ratio [%]
Homogenate	591.6	100
600 × g ppt.	28.8	4.87
12 000 × g ppt.	50.9	8.60
100 000 × g ppt.	107.2	18.12
100 000 × g sup.	404.7	68.41

3-Day-old etiolated hypocotyl tips (32.8 g fresh wt, section I + II, see Fig. 5) were used to the study. Cellular fractionation and enzyme assay were performed as described in Materials and Methods.
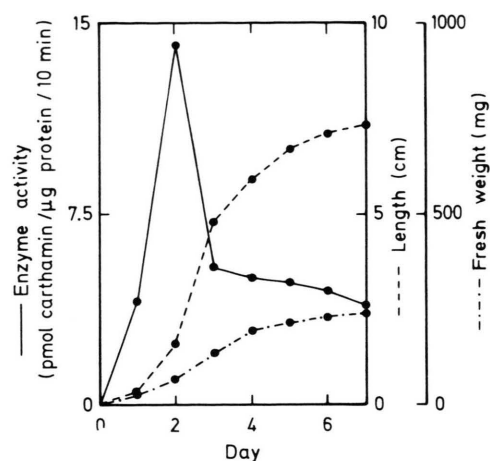

Fig. 4. Enzyme activity in different aged hypocotyls obtained from 1–7 days old seedlings after sowing. Etiolated seedlings used as follows: 1-day-old, 6.5 g; 2-day-old, 10.6 g; 3-day-old, 9.7 g; 4-day-old, 9.7 g; 5-day-old, 10.8 g; 6-day-old, 11.7 g; 7-day-old, 12.1 g. For details of enzyme preparation and enzyme assay see Materials and Methods.
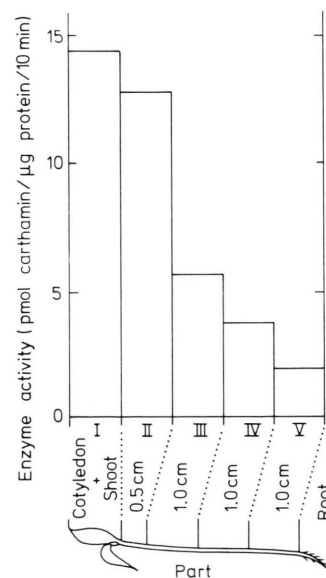

Fig. 5. Enzyme activity in different parts of hypocotyls obtained from 3-day-old seedlings after sowing. Etiolated hypocotyls (22.2 g fresh wt) from 3-day-old seedlings were used for enzyme preparation. Methods for enzyme preparation and enzyme assay were carried out as described in Materials and Methods.

fraction of safflower seedlings (Table II); more than 68% of the enzyme activity was found in $100\,000 \times g$ supernatant fluid which was arised from cytosol of the hypocotyl cells. Carthamin-synthesizing enzyme might occur in the cytosol of the cells of safflower tissues and could play a significant role in the oxygenative catabolism of a flame-coloured precursor to a reddish plant pigment, carthamin.

In the previous study, we showed that a cell-free extract prepared from mature safflower plant could synthesize carthamin under aerobic conditions. The activity of the enzyme distributed in all above-ground parts of the plant examined [1]. In the course of our experiments using florets of safflower at the blooming stage, however, we faced a difficulty in collecting fresh material for enzyme preparation, because the harvest time of petals from the flowering plant is very restricted in a year. The finding reported here that seedlings of safflower contained enzyme responsible for synthesizing carthamin from the precursor may overcome the difficulty, for safflower seedlings are always available. Furthermore, the method for cultivation of the seedlings is relatively easy, studies on carthamin synthesis in *C. tinctrius* will be promoted by using seedlings as the material for enzyme preparation.

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[3] M. Wada, Jap. Pat. 8943 (1955).