Formation of carthamin by a partially purified enzyme from safflower seedlings

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Summary. An enzyme responsible for synthesizing carthamin from precarthamin was partially purified and the catalytic properties were investigated.
Key words. Safflower; carthamin; enzymic synthesis.

A carthamin-synthesizing enzyme plays an important role in the metabolic process in which carthamin is formed from precarthamin, by which color transition is induced in safflower florets at a late stage of blooming. Recently, results from in vitro studies have shown that the carthamin-synthesizing enzyme is widely distributed over the parts of the plant above the ground, but strong activity of the enzyme was restricted to younger developing tissues. Atmospheric oxygen was required for the reaction of carthamin synthesis. The enzyme was labile on storage and extremely sensitive to phosphate ions, which properties prompted us to assume that the enzyme might differe from polyphenol oxidases[3] or peroxidases[4,5]. In an earlier experiment we found that etiolated seedlings of safflower could also serve as a useful source for enzyme preparation. The activity of the carthamin-synthesizing enzyme was found to be localized in the apical area of the hypocotyl, and it was preferentially associated with the cytosol fraction[6].

In this study we isolated the enzyme from the soluble fraction of the etiolated hypocotyl tips and purified it partially. The present paper deals with evidence of carthamin synthesis by the partially purified enzyme preparation. Some properties of the enzyme are also described.

Materials and methods. An authentic carthamin was prepared according to the method of M. Wada (Japan patent No. 8943, 1955). Freshly collected flower florets of safflower (*Carthamus tinctorius* L). were crushed, pressed out and dried in the 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, which were then acidified with 10% (v/v) citric acid. The muslin with the adsorbed carthamin was washed well with water and exhaustively extracted with 60% aqueous acetone. The extracts were evaporated in vacuo at below 30 °C to give a greenish-red mass. The crude carthamin was recrystallized several times in 60% aqueous acetone to give fine micro-needles with a dark-reddish metallic luster. The purified carthamin was used as an authentic specimen for identification of the product of the enzymic reaction. Chemicals used were of the highest grade of purity and obtained from commercial sources. Mushroom tyrosinase (dihydroxyphenylalanine:oxygen oxidoreductase; EC 1.14.18.1) and horseradish peroxidase (donor:hydrogen-peroxide oxidoreductase; EC 1.11.1.7) were purchased from Sigma Chemical Co. (St. Louis, Mo., USA). Protein was estimated by the method of Lowry et al.[7]. Sulfhydryl residues in enzyme protein were detected by the phenazine methosulfate method[8]. UV spectra were registered on a Shimadzu type MPS-2000 spectrophotometer.

All preparative operations were performed in the cold at 2–4 °C as quickly as possible. Freshly collected hypocotyl tips (100 g) were dipped in 2.5 vol./g of ice-cold 70% (v/v) ethanol for 3 min and washed 6 times with 3 vol./g of cold distilled water each time. The washed material was homogenized with ice-cold 50.0 mM citrate-phosphate buffer, pH 7.0, containing 20.0 mM sodium D-araboascorbate and 0.1 mM 2-mercaptoethanol in a pre-chilled Waring blendor. The homogenate (520 ml) was squeezed through three layers of muslin-cloth and centrifuged for 20 min at 15,000 × g. The supernatant (510 ml) was fractionated with solid $(NH_4)_2SO_4$. The protein fraction precipitating between 30 and 60% $(NH_4)_2SO_4$ saturation was collected and dissolved in 50.0 mM citrate-phosphate buffer, pH 7.0, which was passed through a column (3.5 × 17 cm) of Sephadex G-25. The transit liquid was stirred with 0.5% $Ca(CH_3CO_2)_2$ and centrifuged for 20 min at 15,000 × g. The supernatant (71 ml) was stirred for 20 min in 1.5% aqueous protamine sulfate (14 ml) and the precipitate was removed by centrifugation. After Sephadex G-25 gel filtration, the desalted liquid (22 ml) was diluted 2 times by volume with 5.0 mM citrate-phosphate buffer, pH 7.0, and passed through a Celite 535 column (2.4 × 16 cm) previously equilibrated with the same buffer. Fractions (3–10) were combined (81 ml) and the protein was concentrated by $(NH_4)_2SO_4$ precipitation. The pellet was then applied to a column (1.5 × 90 cm) of Sephadex G-100 and eluted with 50.0 mM citrate-phosphate buffer, pH 7.0. Fractions of 5 g were collected and slightly yellowish brown fractions (No. 8–13), with a specific activity greater than 4.9 μunit/μg protein, were retained for the following enzyme assay.

Catalytic enzyme activity was assayed in a colorimeter by measuring the initial increase in absorbance at 517 nm. The assay medium contained, unless otherwise mentioned, 1.0 μg of precarthamin and 0.1 mM dithiothreitol made up to a total volume of 4.0 ml by addition of 50.0 mM citrate buffer, pH 5.2. Incubation was started immediately after mixing the enzyme protein into the incubation mixture. The activity of the enzyme was expressed in terms of specific activity (unit/μg protein; 1 unit = 1 μmol carthamin/min) and was an average value of four determinations. In tests of gases the assay mixture, from which the air had been removed by suction, was separately

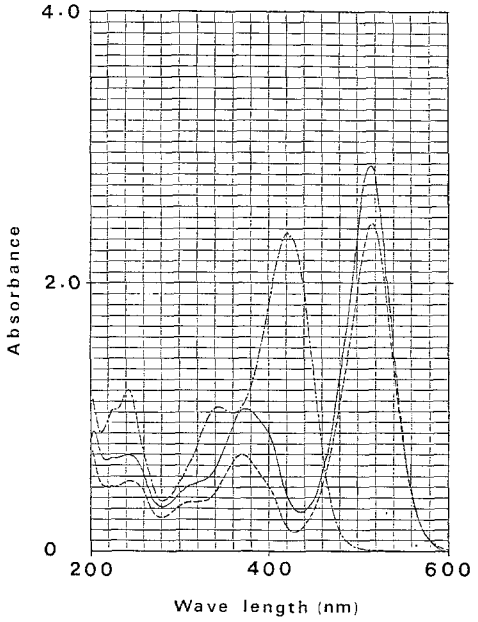

Figure 1. UV spectra of reaction product, authentic carthamin and precarthamin. Each compound was measured in purified methanol at the following concentrations, as indicated (mg/10 ml); ——, reaction product (0.5); – – –, authentic carthamin (0.5); –·–·–·–, precarthamin (0.18).

Effect of gas phases on the activity of a carthamin-synthesizing enzyme

Gas phase	Specific activity (μ unit/ μg protein)	Inhibition (%)
Ar	4.77	8.97
N_2	4.94	5.73
H_2	5.13	2.10

The air in the reaction mixture was removed by suction for 5 min and then each test gas was bubbled separately into the incubation medium for 30 min at 25 °C just before initiation of the enzyme assay. The assay mixture used for the control was bubbled with air for 30 min at 25 °C. Specific activity of the enzyme in the control test was 5.24 μ unit/μg protein. Enzyme activity was measured as described in the text.

bubbled for 30 min with the gas for testing just before enzyme assay initiation. Reactions were performed for 10 min at 30 °C. The activity of polyphenol oxidase was measured spectrophotometrically by following changes in absorbance at 475 nm when 3,4-dihydroxyphenylalanine was supplied as a substrate[9]. The reaction mixture contained 4.0 mM L-3,4-dihydroxyphenylalanine, 222–223 μg partially purified enzyme protein and 100.0 mM phosphate buffer, pH 6.7, in a total volume of 3.5 ml. The color change was measured at 30 °C for 5 min immediately after addition of the enzyme into the reaction mixture. Peroxidase activity was estimated at 420 nm using guaiacol as the hydrogen donor, essentially after the method of

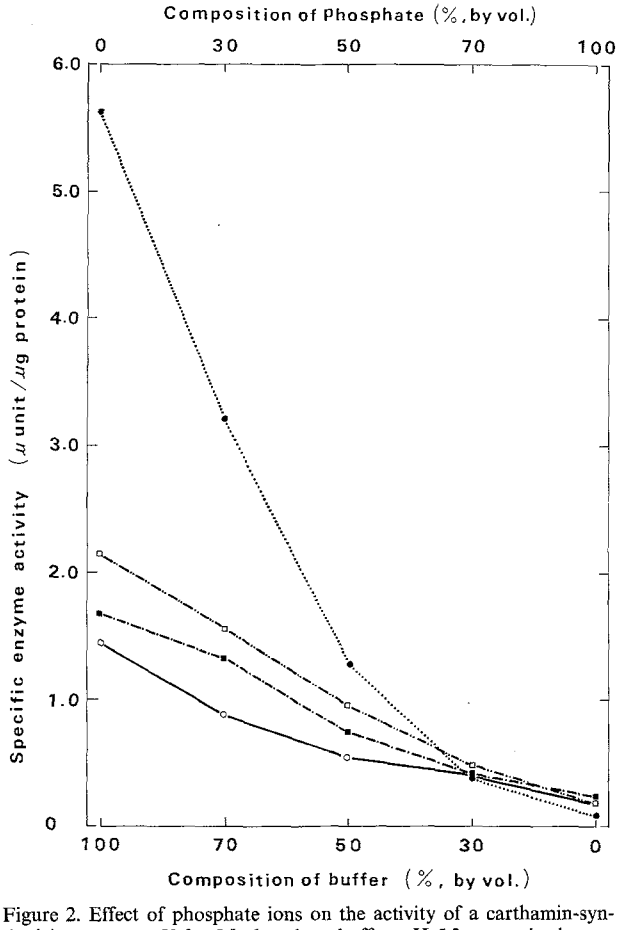

Figure 2. Effect of phosphate ions on the activity of a carthamin-synthesizing enzyme. 50.0 mM phosphate buffer, pH 5.2, was mixed separately with each buffer for testing at the mixing ratio indicated in the figure. ● · · · ●, 50.0 mM acetate buffer, pH 5.2; □–·–·–□, 50.0 mM phthalate buffer, pH 5.2; ■–·–·–■, 50.0 mM succinate buffer, pH 5.2; ○—○, 50.0 mM citrate buffer, pH 5.2. Enzyme assays were done as described in the text.

Tomiyama and Stahmann[10]. The incubation medium was composed of the following components in a final volume of 4.0 ml: 11.0 mM guaiacol, 16.5 mM H_2O_2, 222–223 μg enzyme protein, 55.0 mM phosphate buffer, pH 6.0. The reaction was carried out at 30 °C for 5 min.

Results and discussion. An enzyme which catalyzes carthamin synthesis from precarthamin was extracted from the homogenate of the etiolated hypocotyl tips of safflower seedlings and partially purified through many purification stages. Purification up to 8.7-fold with a recovery of 12% was achieved. The partially purified enzyme could synthesize carthamin from precarthamin. At the optimum pH (5.2) and 30 °C, the rate of carthamin synthesis was estimated to be 8.11 μunits/μg protein under the present assay condition. The purified sample of the reaction product was identified as carthamin by comparing the chromatographic behaviors and the spectrophotometric properties with those of an authentic sample. The R_f values observed by using three different solvent systems coincided well with each other. UV spectra of the enzymic product and a standard carthamin were found to be parallel. A typical result is shown in figure 1. Comparison of the ^{1}H-NMR spectral data were also satisfactory. IR spectra of the reaction product and an authentic carthamin could be superimposed on each other. The solution of the enzyme was slightly yellow brown and displayed an absorption maximum at 278 nm and a weak inflexion between 330 and 440 nm. Emission spectrochemical analysis showed that the enzyme contain Ca, Mg, K, Cu and Fe. A spot test with a reagent specific for -SH groups revealed the presence of a cysteinyl residue in the enzyme protein.

In our previous studies we reported that aerobic conditions were required for the enzyme reaction[2]. Data from experiments concerning the effect of three different gas phases on carthamin synthesis are presented in the table. Deficiency of atmospheric oxygen in the assay mixture strongly depresses the reactivity of the enzyme. These evidences indicate the possibility that the process of carthamin synthesis in safflower tissues is controlled by an enzyme-mediated biooxidation reaction. In an attempt to characterize the catalytic property of the carthamin-synthesizing enzyme, we tested whether the enzyme preparation would show polyphenol oxidase or peroxidase activity. However, only negative results could be obtained from the studies. These findings clearly contrast with the data of Shimokoriyama and Hattori[11]. These authors used the yellow ethanol powder obtained by ethanol extraction of *Carthamus* flowers as the enzyme preparation for their in vitro studies. In our preliminary observations we found that besides the activity of the carthamin-synthesizing enzyme, both polyphenol oxidase and peroxidase activities are always detected in the ethanolic floret extract of safflower. These three activities could not be separated satisfactorily from the extract by applying the methods of ethanol or acetone precipitation. The procedures employed for the present enzyme purification practically abolished both polyphenol oxidase and peroxidase activities from the carthamin-synthesizing enzyme preparation.

The sensitivity of the carthamin-synthesizing enzyme to phosphate ions, which had been found in previous studies[2,6], was examined again more precisely. Enzyme assays were carried out in 50.0 mM buffers, pH 5.2, with varying concentrations of phosphate in the buffer systems. The results prove that phosphate ions have strong inhibitory action on the enzyme activity (fig. 2). The rate of synthesis of carthamin from its precursor gradually decreases in proportion to the increasing amount of phosphate ions in citrate, succinate, acetate and phthalate buffer solutions. These facts strongly support the results drawn from earlier investigations[2,6] and at the same time they suggest the possibility that the carthamin-synthesizing enzyme may be a new type of polyphenol oxidizing enzyme. However, further studies are necessary for characterizing the catalytic properties of the enzyme.

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Dopamine-beta-hydroxylase activity in adrenal gland and spleen of rats after fasting and cold exposure*

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Summary. Fasting (48 h) results in dopamine-beta-hydroxylase (DBH) release both in adrenal gland and spleen, suggestive of an increase in the activity of these organs. Cold exposure (48 h) produces a dissociation of the sympathoadrenal response. When both stimuli are simultaneously employed, the DBH response suggests the preponderance of the response to fasting. Plasma DBH is decreased in all groups studied, this could be due to its half-life and the splenic DBH depletion.
Key words. Rat, adrenal; rat, spleen; adrenal, rat; spleen, rat; fasting; cold exposure; dopamine-beta-hydroxylase.

The sympathetic nervous system and adrenal medulla regulate physiological responses to stress and adverse environmental conditions. The secretion of catecholamines and noradrenaline-forming enzyme, dopamine-beta-hydroxylase (DBH) occurs by exocytosis[2]. Several studies have attempted to demonstrate that DBH activity could serve as an index of sympathetic activity[3,4]. In the present study, we have evaluated whether DBH activity in rat adrenal gland, spleen and plasma could be modified by short-term cold exposure and brief fasting.

Material and methods. Male Wistar rats (300–350 g) were used in all experiments. All animals were housed in individual cages for 48 h and divided into 4 groups: 1) Control (n = 8); 2) cold exposure (n = 8): 48 h in a cold room at 4 °C; 3) fasting (n = 8) and 4) fasting plus cold exposure (n = 8). After 48 h, rats were anesthetized with ethyl ether and 1 ml of heparinized blood was obtained by cardiac puncture. Blood samples were collected in chilled tubes and spun at 5000 × g for 10 min at 4 °C. The plasma was stored at −20 °C until it was used for DBH assay. 0.1 ml of blood was used for the glucose determination, as described by Werner et al.[5]. Adrenal glands and spleen were removed and homogenized with an ice-cold 5 mM Tris-HCl solution, pH 7.3, containing 0.2% Triton X-100; after centrifugation (26,000 × g for 10 min), 5-µl (adrenal) and 10-µl (spleen) portions of the supernatants were taken for DBH assay[5]. DBH activity in plasma and in tissues was assayed by a sensitive procedure using tyramine as substrate[4]. In order to obtain an optimal enzymatic activity and overcome the effect of endogenous inhibitors[7], the following copper concentrations were selected: 16.6 µm/tube (adrenal), 33.3 µm/tube (spleen) and 47.6 µm/tube (plasma). In the DBH assay, the optimal plasma volume and the optimal tyramine concentration were 25 µl and 0.645 mM, respectively. The concentration of octopamine standards was 0.2 nm/tube. All samples, blanks and standards were assayed in duplicate in the cold (4 °C). The variability between replicates was 5.6% for adrenal, 4.9% for spleen and 4.1% for plasma. The adequate inactivation of enzyme inhibitors was further tested by adding to a duplicate of each sample a known amount of a partially purified bovine adrenal DBH. Using these aliquots of tissue homogenates and plasma, the recoveries were always greater than 90%; data were not corrected for recoveries. To evaluate the significance of a difference between mean values, Student's t-test was used. A p-value < 0.05 was considered to indicate a significant difference.

Results and discussion. In the present work DBH activity was studied in rat adrenal gland, spleen and plasma after cold exposure and/or fasting for 48 h. The spleen was chosen because of its rich sympathetic innervation.

Fasting for 48 h produces a significant decrease in DBH content of both organs (table) as a result of DBH release, suggesting an increase in the activity of these organs. It is well established that hypoglycemia increases the activity of adrenal medulla[8]. Therefore, the significant decrease of plasma glucose levels in this group (table) could be partly responsible for the increase in adrenal release of DBH. Plasma DBH activity, after fasting, is significantly decreased compared to control (table). This situation can be explained by the following observations; first, DBH in plasma is derived mainly from the sympathetic nerves rather than the adrenal medulla[9]; second, some stimuli that produce activation of the sympathoadrenal system cause

	DBH activity			
	Adrenal	Spleen	Plasma	Plasma glucose
Control	1.368 ± 0.196	0.114 ± 0.004	9.715 ± 0.478	103.670 ± 2.210
Cold exposure	2.306* ± 0.220	0.066* ± 0.007	7.819* ± 0.286	117.980* ± 4.250
Fasting	0.875* ± 0.083	0.015* ± 0.002	6.450* ± 0.290	66.240* ± 1.440
Cold exposure plus fasting	0.899* ± 0.064	0.036* ± 0.0035	6.730* ± 0.150	60.840* ± 1.550

The results are expressed as the mean ± SEM for 8 animals. DBH activity is expressed in nmoles of octopamine/mg of tissue (adrenal and spleen) and nmoles of octopamine/ml of plasma/h. Plasma glucose is expressed as mg/100 ml. * $p < 0.05$ compared to control.