Preparation of an Enzyme Associated with Carthamin Formation in *Carthamus tinctorius* L.

Koshi Saito, Yoshiyuki Takahashi, and Mizu Wada

Department of Applied Chemistry, Faculty of Engineering, Tokai University, 1117 Kitakaname, Hiratsuka-shi, Kanagawa-ken 259-12, Japan

Z. Naturforsch. **40c,** 819–826 (1985); received August 12, 1985

Carthamin Synthesis, Safflower Seedling, Catalytic Property

An enzyme associated with carthamin formation in *Carthamus tinctorius* L. (carthamin-synthesizing enzyme) was isolated from the soluble protein extract of the hypocotyl tips of the etiolated seedlings and purified up to 157-fold by the procedures applying $(NH_4)_2SO_4$ fractionation, $Ca(CH_3CO_2)_2$ precipitation, protamine sulfate treatment, Celite adsorption chromatography, and Sephadex G-100 gel filtration. Results from atomic absorption spectral analysis of the enzyme protein showed to contain K as a major component, and Ca and Mg as minor ones. Fe, Cu, and Mn could not be detected in the preparation. At pH 4.8 in 50.0 mM acetate buffer, the partially purified enzyme reacted positively with a flame-coloured precarthamin to produce a reddish product in open cuvettes with incubation medium. The reaction product was identified as carthamin by examining its colour, chromatographic mobilities in different developing solvents and spectroscopic properties inclusive shifts, often by comparing with those of an authentic specimen. Anaerobic incubation reduced the enzyme activity, while exogenously applied O_2 slightly enhanced the catalytic rate of carthamin formation. The enzyme was sensitive to phosphorus substances. Among those compounds tested at 1.2 mM level, orthophosphate showed the most striking inhibitory action on the enzyme. Metal ions affected on the enzyme activity by different extents. Mn^{2+} stimulated the enzyme reaction, while Cu^{2+} and Mo^{6+} exhibited reverse effects. Fe^{2+}, Fe^{3+}, Zn^{2+}, Mg^{2+}, and Co^{2+} were also unfavourable to the enzyme catalyzed carthamin formation. The preparation of the carthamin-synthesizing enzyme showed no activity of polyphenol oxidase or peroxidase under the conditions specifically designed for detecting both enzyme activities.

Introduction

Carthamin, a red quinochalconoid plant pigment, is usually detectable in matured *Carthamus* florets. At the late stage of blooming, carthamin formation is rapidly induced by an enzyme distributed over vegetative parts of the flowering plant. In recent studies we found that the enzyme was localized mainly in the cytosol of younger developing tissues of safflower and preferentially active under aerobic conditions rather than anaerobic [1–3]. These facts suggest that the enzyme plays an integral role in the process of oxidative metabolism of a flame-coloured precarthamin leading to the formation of red carthamin within safflower florets.

Previously, attempts to synthesize carthamin *in vitro* systems were simultaneously undertaken in two different laboratories [4–6]. Thus, an assumptive mechanism was proposed for the enzyme reaction, suggesting that polyphenol oxidase- or peroxidase-catalyzed oxidation of a leucopigment might be involved in the process of reddening of yellow *Carthamus* flowers [6]. On the other hand, it has been noted in our previous observations that the enzyme responsible for carthamin formation (carthamin-synthesizing enzyme) is very sensitive to phosphate ions [1–3]. The observed instability against phosphate was quite unexpected, since phosphate buffer has routinely been applied to the extraction and assay of many oxidases including polyphenol oxidases (monophenol monooxygenase) and peroxidases. Therefore, it seems to be of interest to compare the catalytic properties of our enzyme preparation with those of polyphenol oxidases or peroxidases. As the extract of the carthamin-synthesizing enzyme from *Carthamus* tissues is always contaminated with native polyphenol oxidases and peroxidases, and moreover, it is very unstable in air our initial attempts to purify the crude extract of the carthamin-synthesizing enzyme were not successful.

In this study we purified partially the carthamin-synthesizing enzyme sample from the soluble protein extract of safflower seedlings and examined some

Enzymes: monophenol monooxygenase or dihydroxyphenylalanine:oxygen oxidoreductase (EC 1.14.18.1); peroxidase or donor:hydrogenperoxide oxidoreductase (EC 1.11.1.7).

Reprint requests to Dr. K. Saito.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/85/1100–0819 $ 01.30/0

catalytic properties of the enzyme in relation to study on the mechanism of carthamin formation in *Carthamus* flowers.

Materials and Methods

Chemicals

Commercial chemicals used were obtained from the following sources: Sephadex G-100 and blue dextran (Pharmacia Fine Chemicals; Uppsala, Sweden), Celite No. 535 and ADP (Tokyo Kasei Kogyo Co., Ltd.; Tokyo, Japan), 2-mercaptoethanol (Kanto Chemical Co., Inc.; Tokyo, Japan), ATP (Wako Pure Chemical Ind.; Osaka, Japan), AMP and IMP (Kohjin Co., Ltd.; Tokyo, Japan), polyphenol oxidase (mushroom), peroxidase (horseradish) and protamine sulfate (Sigma Chemical Co.; St. Louis, Mo., USA). All other chemicals used were of reagent grade.

Plant material

Seeds of safflower (*C. tinctorius* L.) were germinated on moist vermiculite at 27 °C in the dark. Etiolated hypocotyl tips (1044 g fresh wt.) were harvested from 3–4 days old seedlings after sowing and dipped in 3.3 ml per g fresh weight of 70% aqueous ethanol for 3 min, followed by washing 6 times with 5.0 ml per g fresh weight of distilled water to remove residual ethanol.

Preparation and purification of the extract of a carthamin-synthesizing enzyme

The washed hypocotyl tips were homogenized with a pre-chilled Waring blendor in 800 ml of ice-cold 50.0 mM phosphate buffer, pH 7.2, containing 20.0 mM sodium D-araboascorbate and 0.1 mM 2-mercaptoethanol. The homogenate was squeezed through two layers of nylon-cloth and the filtrate centrifuged at $15000 \times g$ for 20 min. The supernatant was brought to 40% saturation by addition of solid $(NH_4)_2SO_4$ with continuous stirring in an ice-bath. The mixture was allowed to stand at 2–4 °C for 20 min and then centrifuged at $15000 \times g$ for 10 min. The supernatant was brought further to 80% saturation with $(NH_4)_2SO_4$, and the precipitate collected in the same way and dissolved in a minimum volume of 50.0 mM phosphate buffer, pH 7.2, then passed through Sephadex G-25 column (3.5×60 cm). The salt-free protein solution (414 ml) was treated with 2.1 g of $Ca(CH_3CO_2)_2$. After standing for 20 min with gentle stirring, the mixture was centrifuged at $15000 \times g$ for 20 min to remove the precipitate. To 393 ml of the supernatant liquid, 1.5% (w/v) aqueous neutralized solution of protamine sulfate (55.1 ml) was added dropwise with continuous stirring and resulting precipitate removed by centrifugation at $15000 \times g$ for 20 min. The supernatant (444 ml) was made up to 80% saturation with solid $(NH_4)_2SO_4$ and the pellet collected, then dissolved in 5.0 mM phosphate buffer, pH 7.2, and residual salt removed by gel filtration through Sephadex G-25 in the same buffer. An aliquot of the transit liquid was passed through a Celite 535 column (2.7 × 71 cm) and adsorbed protein eluted with 5.0 mM phosphate buffer, pH 7.2. The protein in the eluate was concentrated by addition of solid $(NH_4)_2SO_4$ up to 80% saturation. It was then dissolved in a small amount of 50.0 mM phosphate buffer, pH 7.2, and an aliquot passed through a column (0.9×90 cm) of Sephadex G-100 in the same buffer. The active fraction, eluted between 40 and 60 ml (fraction nos. 8–11, each 5 ml vol.) were retained for enzyme assay and atomic absorption spectral analysis.

Preparation of a sample for the atomic absorption spectral analysis of the enzyme protein

A portion of the enzyme solution obtained from the final purification step of Sephadex G-100 gel filtration was brought to 80% saturation with $(NH_4)_2SO_4$. The pellet was collected, dissolved in 15 ml of 50.0 mM phosphate buffer, pH 7.2, and passed through a column (1.5×30 cm) of Sephadex G-25, which was suspended in distilled water. The transit liquid (20 ml) was packed in a semipermeable membrane tube and dialysed by continuous shaking in an incubator (Yamato, type BT-46, 90 strokes/min) at 7–8 °C for 5 h in distilled water (2000 ml) containing 1.0 M EDTA. The dialysis was continued further for about 20 h: the water was replaced at intervals by freshly distilled water (6000 ml in total), then exchanged anew redistilled water (4000 ml in total) which prepared by boiling with $KMnO_4$ for about 24 h. At the end of the dialysis, the protein solution was transferred to a flask and freeze-dried under reduced pressure with liquid nitrogen. Dried protein powder (103.1 mg) was burned into ashes on a platinum boat, which was dissolved in 2.0 ml of conc. HCl, followed by being diluted with distilled water to be 25 ml. This sample was used for the process of the atomic absorption spectral analysis. A platinum boat

without dried protein powder was burned in the flame and washed with 2.0 ml conc. HCl. The combined washings were then diluted with distilled water to be 25 ml, whose solution was used to the atomic absorption spectral analysis of the purified enzyme sample as the reference.

Assay of the enzyme activity

Two methods, direct and indirect, were employed for estimation of the carthamin-synthesizing enzyme activity. The former was determined spectrophotometrically, essentially after the method reported in our previous communications [1–3]. Carthamin formed by the enzyme reaction was followed with a spectrophotometer at 517 nm (Shimadzu, type MPS-2000). The reaction mixture contained, if not otherwise stated, 160 ng precarthamin and 141–246 μg enzyme protein made up to a total volume of 4.0 ml by addition of 50.0 mM acetate buffer, pH 4.8. The reaction was performed for 2–5 min at 30 °C immediately after mixing enzyme protein into the reaction mixture. The latter was conducted by a cellulose powder adsorption method. The incubation medium composed of 100 mg cellulose powder, 440 ng precarthamin, 284–676 μg enzyme protein and 1.0 ml of 50.0 mM acetate buffer, pH 4.8, in a final volume of 1.5–2.0 ml. The reaction was carried out for 2 h at 30 °C just after addition of enzyme solution into the medium. The enzyme reaction was stopped by 1.0 ml glacial acetic acid and the powder was washed with 5.0 ml distilled water. Then the reddish product was eluted from the cellulose with 10.0 ml of 60% aqueous acetone and the content was estimated from the data obtained by measuring the absorption peak at 517 nm. The activity of the enzyme was expressed as the amount that catalyzed the formation of 1 μmol carthamin per min under the standard assay condition and the specific activity as the activity per mg protein. For specific experiments, anaerobic conditions were obtained in Thunberg cuvettes that carried a side-arm, by repeated evacuation with a two stage high vacuum pump alternated with gas flash. Impurities in the gases were carefully removed by usually employed procedures. The activity of polyphenol oxidase was measured spectrophotometrically by following changes in absorbance at 475 nm when 3,4-dihydroxyphenylalanine was fed as a substrate [7]. Peroxidase activity was estimated at 420 nm using guaiacol as the hydrogen donor [8]. The protein content was determined by the Lowry *et al.* method [9].

Confirmation of the enzymic product

Methods for the isolation and purification of the enzymic product were basically the same as reported in a previous paper [1]. The reaction product was resolved either by thin-layer chromatography on silica gel plates and/or spectrophotometric methods. For the former, the developing solvents used were (by vol.): A. *n*-butanol/acetic acid/water (4:1:2), B. phenol/acetic acid/water (40:1:10), C. ethylacetate/pyridine/water (14:1:10).

Results

Purification of an enzyme associated with carthamin formation

An enzyme associated with carthamin formation in *C. tinctorius* was extracted from the homogenate of the etiolated hypocotyl tips of the seedlings and par-

Table I. Purification of the enzyme responsible for carthamin formation in *C. tinctorius*.

Step of purification	Total volume [ml]	Total activity [mU]	Total protein [mg]	Specific activity [mU/mg protein	Yield [%]	Purification [-fold]
1. Supernatant of crude extract	451	3711.8	25523.0	0.15	100	1.0
2. $(NH_4)_2SO_4$ fractionation (41–80% saturation)	140	406.6	2329.9	0.18	11.0	1.2
3. $Ca(CH_3CO_2)_2$ precipitation	133	449.8	1299.7	0.35	12.1	2.4
4. Protamine sulfate treatment	150	564.9	1227.6	0.46	15.2	3.2
5. Celite 535 chromatography	120	53.8	3.2	16.81	1.5	112.1
6. Sephadex G-100 gel filtration	20	32.9	1.4	23.50	0.9	156.7

Enzyme activity in the purification steps 1, 3, and 4 was measured after filtration of the supernatant through a column of Sephadex G-25.

tially purified through various purification processes. The progress of the purification is listed in Table I. The result shows that the initial purification step by solid $(NH_4)_2SO_4$ fractionation yielded an extract which was purified 1.2-fold over the starting extract. Although no activity of the carthamin-synthesizing enzyme could be separated from those of polyphenol oxidase and peroxidase, innert protein was partially removed by this salting out method. $(NH_4)_2SO_4$ precipitate obtained between 41–80% saturation was treated successively with $Ca(CH_3CO_2)_2$, protamine sulfate, and Sephadex G-25 as described in Materials and Methods. The salt-free protein fraction was placed directly on a Celite column and chromatographed by gravity flow with 5.0 mM phosphate buffer, pH 7.2. Fig. 1 shows the elution profiles of the

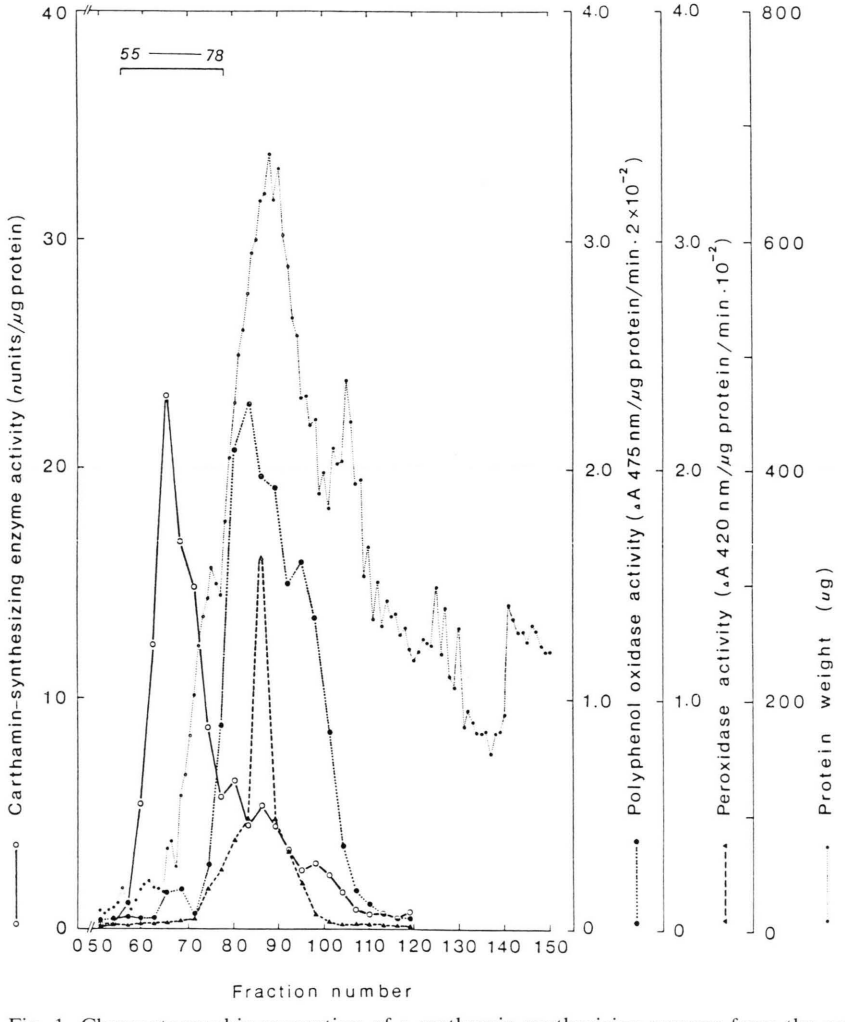

Fig. 1. Chromatographic separation of a carthamin-synthesizing enzyme from the activities of polyphenol oxidases and peroxidases on a Celite column. Salt-free protein solution from the purification step 4 was passed through a column (2.7 × 71 cm) of Celite 535 which had been previously equilibrated with 5.0 mM phosphate buffer, pH 7.2, and the adsorbed protein eluted with the same buffer. The activity of a carthamin-synthesizing enzyme was measured under the standard assay condition. The activities of polyphenol oxidases were measured spectrophotometrically according to ref. [7] in 100.0 mM phosphate buffer, pH 6.7, containing 4.0 mM L-3,4-dihydroxyphenylalanine in a final volume of 3.5 ml. The colour change, manifested by increasing absorbance at 475 nm, was recorded at 30 °C for 5 min. Peroxidase activities were assayed spectrophotometrically by recording the change in optical density at 420 nm within 5 min at 30 °C. The reaction mixture contained following components in a total volume of 4.0 ml; 11.0 mM guaiacol, 16.5 mM hydrogen peroxide, 55.0 mM phosphate buffer, pH 6.0. The bar in the figure shows collected fractions for following purification process of the carthamin-synthesizing enzyme by Sephadex G-100 gel filtration.

enzyme protein and the activities of the carthamin-synthesizing enzyme, polyphenol oxidase, and peroxidase. As seen in the Fig. 1, the activity of the carthamin-synthesizing enzyme could be separated satisfactorily from both native polyphenol oxidase and peroxidase activities at this purification step. Combined active fractions (fraction nos. 55–78) of the carthamin-synthesizing enzyme were concentrated by $(NH_4)_2SO_4$ and filtered through a column of Sephadex G-100. It exhibited a clear resolved pattern obtained as a symmetric peak when the enzyme was chromatographed on the column. At this step about 160-fold purification of the enzyme was accomplished (see Table I). The purified enzyme protein was subjected to the atomic absorption spectral analysis, which revealed that the enzyme contained K most predominantly. Ca and Mg came next, however, no Cu, Fe, and Mn were detected in the preparation (Table II).

Table II. Metal content in a partially purified enzyme fraction.

Metal	Content [ng]
Cu	–*
Fe	–*
Mn	–*
K	85.0 ± 0.4
Ca	22.5 ± 0.1
Mg	23.5 ± 0.2

* Not detected. Preparation of the enzyme protein and the reference sample was shown in the text.

Catalytic properties of the carthamin-synthesizing enzyme

Optimum pH for the enzyme reaction. The optimum pH for conversion of a flame-coloured substrate, precarthamin to red carthamin by the purified enzyme sample was tested in 50.0 mM acetate buffer with different pH values. The highest activity was observed at pH 4.8, whose value is lower than that of reported previously [1, 2]. The rate of the enzyme reaction at pH 4.8 in both acetate and citrate buffers was almost constant over the first 15 min and then it progressively declined.

Oxygen requirement for the enzyme reaction. Oxygen requirement for the reaction is suggested by relating the amount of carthamin formed in the presence or absence of air. The affinity of the enzyme for oxygen, determined by its maximal activity under aerobic or anaerobic conditions with various gas phases is shown in Table III. The enzyme requires atmospheric oxygen for the optimal reaction. In order to characterize the catalytic property of the carthamin-synthesizing enzyme, we examined at first if it would show polyphenol oxidase or peroxidase activity. The results are illustrated in Figs. 2 and 3.

Table III. Effect of various gas phase on the activity of a carthamin-synthesizing enzyme.

Gas phase	Specific activity [μunits/μg protein]	Rest of enzyme activity [% of the control]
Ar	8.85 ± 0.14	89.6
N_2	9.13 ± 0.19	94.8
H_2	9.58 ± 0.06	99.5
O_2	10.12 ± 0.22	105.1

Specific activity of the enzyme in the control test was 9.63 μunits/μg protein in average of three different determinations.

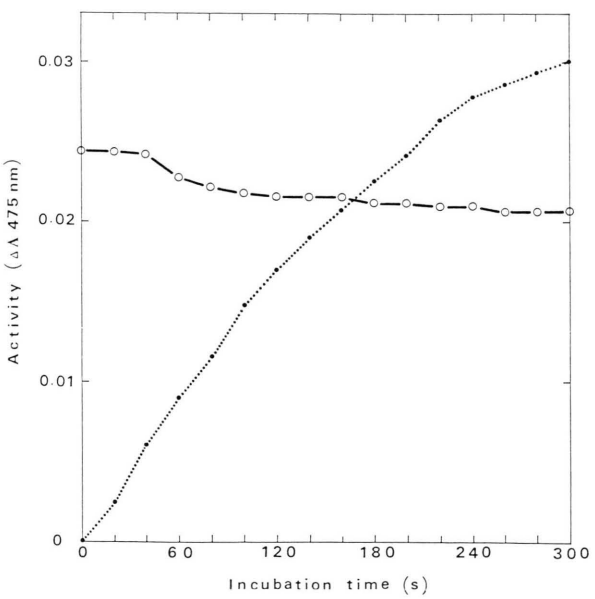

Fig. 2. Comparison of the catalytic activity of a carthamin-synthesizing enzyme with that of mushroom polyphenol oxidase. 6500 mUnits mushroom polyphenol oxidase (2600 units/mg solid) were used in this study. The enzyme assays were done as described in Materials and Methods and Fig. 1. Carthamin-synthesizing enzyme activity (○———○) and mushroom polyphenol oxidase activity (●·····●).

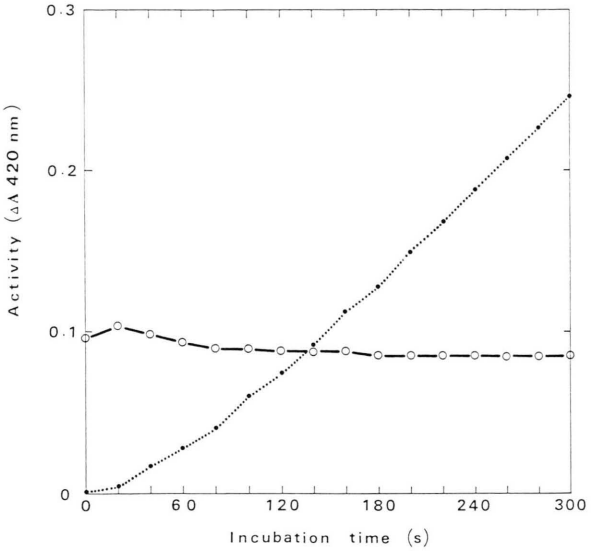

Fig. 3. Comparison of the catalytic activity of a carthamin-synthesizing enzyme with that of horseradish peroxidase. 15.5 mUnits horseradish peroxidase (265 purpurogallin units/mg solid) were used in the present investigation. The activities of the enzymes were determined as shown in Materials and Methods and Fig. 1. Carthamin-synthesizing enzyme activity (○———○) and horseradish peroxidase activity (●·····●).

Effect of phosphorus compounds on the enzyme activity. The sensitivity of the carthamin-synthesizing enzyme to phosphate ions was suggested by the results from earlier investigations [1–3]. In this study the effect of various phosphorus compounds on the enzyme was reexamined more precisely (Table IV). Catalytic activity of the enzyme was affected by many phosphorus substances. Among those compounds tested at 1.2 mM level, orthophosphate showed the most striking inhibitory effect on the enzyme, while meta-, pyro-, and poly-phosphate brought about inhibition by 23.3, 2.7, and 1.1%, respectively. Potassium salts restricted the enzyme reaction more strongly than those of sodium. Bibasic salts were less preferable for the carthamin formation than monobasic ones (see Table IV).

Effect of metal ions on the activity of the enzyme. The effect of metalic ions on the enzyme activity was tested at various concentrations (Table V). At 0.012–0.6 mM level, only Mn^{2+} conspicuously enhanced the catalytic enzyme activity. Cu^{2+} strongly inhibited the enzyme reaction at all the concentrations examined. The enzyme action was seriously reduced by Mo^{6+} at the concentrations ranging from

Table IV. Effect of phosphorus compounds on the activity of a carthamin-synthesizing enzyme from safflower seedlings.

Supplement*	Specific activity [μunits/μg protein]	Rest of activity [% of the control]
None	7.09	100
IMP	4.83	68.12
AMP	4.81	67.84
ADP	3.97	55.99
ATP	2.88	40.62
P_2O_5	3.37	47.53
H_3PO_4	0.03	0.42
HPO_3	4.46	62.91
H_3PO_3	5.44	76.73
$H_4P_2O_7$	6.90	97.32
$H_6P_4O_{13}$	7.21	98.90
K_2HPO_4	2.47	34.84
KH_2PO_4	3.99	56.28
Na_2HPO_4	5.35	75.46
NaH_2PO_4	5.78	81.52

* Each compound was tested at 1.2 mM.

0.06 to 1.2 mM. Other metal ions such as Fe^{2+}, Fe^{3+}, Mg^{2+}, Zn^{2+}, and Co^{2+} also exhibited different inhibitory effects on the enzyme.

Proof of the enzymically synthesized carthamin

The reaction product was identified as carthamin by ascending chromatography of the sample from the reaction mixture on silica gel plates using three different solvent systems as described above. The R_f values observed were (A–C): reaction product = 0.46, 0.47, 0.28; authentic carthamin = 0.46, 0.47, 0.30. UV spectrum of the purified enzymic product showed its absorption maxima at 244 (ε 14000), 310 (ε 9400), 347 (ε 20000), and 517 nm (ε 53000) in methanol. UV absorption spectrum of the reaction product taken after addition of a few drops of $AlCl_3$ solution showed a bathochromic shift of 49 nm (λ_{max} 517 → 468 nm). Another fact was also confirmed by examining the UV spectrum of the product, which gave a shift of 35 nm (λ_{max} 517 → 482 nm) in methanol solution containing CH_3CO_2Na. These results were all coincident well with those of an authentic carthamin.

Discussion

An enzyme associated with carthamin formation in *C. tinctorius* (carthamin-synthesizing enzyme) was isolated from the soluble protein extract of hypocotyl tips of the etiolated seedlings and partially purified

Table V. Effect of metal ions on the activity of a carthamin-synthesizing enzyme from safflower seedlings.

Supplement*	Concentration [mM]	Specific activity [μunits/μg protein]	Rest of activity [% of the control]
None	–	8.20	100
Fe^{2+}	0.012	2.98	36.34
	0.060	2.42	29.51
	0.120	2.13	25.98
	0.600	1.23	15.00
	1.200	0	0
Fe^{3+}	0.012	3.19	38.90
	0.060	2.26	27.56
	0.120	1.80	21.95
	0.600	0	0
	1.200	0	0
Cu^{2+}	0.012	0.07	0.85
	0.060	0.07	0.85
	0.120	0	0
	0.600	0	0
	1.200	0	0
Zn^{2+}	0.012	4.53	55.24
	0.060	3.79	46.22
	0.120	3.59	43.78
	0.600	3.41	41.58
	1.200	0.81	2.20
Mn^{2+}	0.012	16.05	195.73
	0.060	14.79	180.37
	0.120	13.75	167.68
	0.600	9.20	112.20
	1.200	7.04	85.85
Mg^{2+}	0.012	5.05	61.59
	0.060	4.78	58.29
	0.120	4.62	56.34
	0.600	4.30	52.44
	1.200	4.08	49.75
Co^{2+}	0.012	4.60	56.10
	0.060	4.41	53.78
	0.120	4.36	53.17
	0.600	2.32	28.29
	1.200	1.74	21.22
Mo^{6+}	0.012	4.62	56.34
	0.060	0.10	1.22
	0.120	0	0
	0.600	0	0
	1.200	0	0

* Acetate salts were used except for $(NH_4)_6Mo_7O_{24}$ $4H_2O$.

by applying many purification procedures. The enzyme preparation contained potassium most prominantly, though the functional role in the enzyme remains obscure. No heavy metals such as Cu, Fe, and Mn could be found in the preparation. The enzyme catalyzes a stoichiometric conversion of a flame-coloured precarthamin to reddish carthamin in the incubation medium. Replacing air in the incubation cuvettes with Ar, N_2, or H_2 caused in depression of the enzyme activity. While the cuvette was filled with O_2, the catalytic activity of the enzyme raised evidently. This indicates that oxygen may play a role in the metabolic process of precarthamin leading to the formation of carthamin. Shimokoriyama and Hattori reported that polyphenol oxidase- or peroxidase-catalyzed oxidation might be involved in carthamin formation [6], however, it is obviously less prudent to postulate easily the synthetic mechanism of carthamin only because ethanol treated yellow powder of safflower florets turned to red in bufferized solutions, for the activity of the carthamin-synthesizing enzyme is easily detectable in the extract of the safflower tissues along with those of polyphenol oxidases or peroxidases. In the present study we obtained the carthamin-synthesizing enzyme almost free from the activities of the polyphenol oxidases and peroxidases through column chromatographies on Celite and Sephadex G-100. We can prove now that our enzyme preparation differs clearly from polyphenol oxidases and peroxidases, judging from its catalytic properties.

It seems to be worth while to compare some specificities of our enzyme with those of the polyphenol oxidases or peroxidases. The carthamin-synthesizing enzyme requires atmospheric oxygen for the reaction and is really associated with the formation of a polyphenolic plant pigment, carthamin. However, it contains no Cu or Fe as polyphenol oxidases or peroxidases. It shows no activities of polyphenol oxidases and peroxidases. The carthamin-synthesizing enzyme is easily inactivated by a low concentration of orthophosphate and enhanced by the presence of Mn^{2+}. The inhibition by orthophosphate seems not to be exerted through alteration in the ionic environment of the reaction mixture. It may attack directly the active site of the enzyme, since non-enzymic formation of carthamin in bufferized solutions containing precarthamin and oxidizing agents was affected by phosphate ions only slightly [10]. Two roles of the manganese action on the enzyme are possible: manganese could be involved in maintaining the structure of the active enzyme, or it could be directly involved in the catalytic mechanism. By analogy with other oxidases the latter possibility seems a reasonable one. Although it seems

probable that the manganese acts as Mn^{2+}, additional experiments are required to confirm the valence state and to elucidate the mechanism of its participation in the reaction.

Above results confirm us to conclude that carthamin formation in *C. tinctorius* is mediated by a different kind of oxidizing enzyme from polyphenol oxidases or peroxidases. At the late stage of blooming the carthamin-synthesizing enzyme may be activated by supplying a flame-coloured substrate, precarthamin, which reflects rapid accumulation of a reddish product, carthamin, through the process of which colour transition is induced in the safflower florets.

Acknowledgements

We wish to thank Prof. A. Saito, Faculty of Science, Tokai University, for generously performing the atomic absorption spectral analysis of the enzyme protein used in this study. The authors gratefully acknowledge Prof. M. Sasaki, Institute of Research and Development, Tokai University, for offering to use a Shimadzu MPS-2000 spectrophotometer.

[1] K. Saito, Y. Takahashi, and M. Wada, Biochim. Biophys. Acta **756,** 217–222 (1983).
[2] K. Saito, Y. Takahashi, and M. Wada, Z. Naturforsch. **38c,** 724–729 (1983).
[3] K. Saito, Y. Takahashi, and M. Wada, Experientia **41,** 59–61 (1985).
[4] M. Wada, Proc. Japan Acad. **29,** 218–219 (1953).
[5] M. Wada, Proc. Japan Acad. **29,** 351–352 (1953).
[6] M. Shimokoriyama and S. Hattori, Arch. Biochem. Biophys. **54,** 93–101 (1955).
[7] T. Katan and E. Galun, Analyt. Biochem. **67,** 485–492 (1975).
[8] K. Tomiyama and M. A. Stahmann, Plant Physiol. **39,** 483–490 (1964).
[9] O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, J. Biol. Chem. **193,** 265–275 (1951).
[10] K. Saito and Y. Takahashi, Acta Soc. Bot. Pol. **54,** in press.