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Studies on the formation of carthamin in buffer solutions containing precarthamin and oxidizing agents

KOSHI SAITO, YOSHIYUKI TAKAHASHI

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

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Abstract

Formation of carthamin was investigated in bufferized solution containing precarthamin and oxidizing agents. $KMnO_4$, H_2O_2 and HIO_4 were stimulators for carthamin formation. $K_2Cr_2O_7$, MnO_2, Ag_2O, SeO_2 and CrO_3, on the other hand, showed little or no activity in the catalytic carthamin synthesis. $KMnO_4$ was very sensitive to precarthamin, while both H_2O_2 and HIO_4 were insensitive. The optimal pH value for carthamin formation by $KMnO_4$ was 5.3 in 50.0 mM citrate buffer. Antioxidants such as hydroquinone, L-ascorbic acid and D-araboascorbic acid completely inhibited the oxidative conversion of precarthamin to carthamin at a low concentration.

Key word: carthamin, carthamin formation, oxidizing agents

INTRODUCTION

One of the most attributive characteristics of safflower is its red colouration which appears in yellow florets at the late stage of blooming. This is due mainly to a bichaloconoid type of plant pigment, carthamin, as identified by several workers (Takahashi et al. 1982, Obara and Onodera 1979, Saito et al. 1983a). The synthetic reaction of carthamin is controlled by an enzyme or enzyme system functioning under aerobic conditions (Saito et al. 1983a, b).

In the course of studies on the characterization of a biogenetic carthamin precursor, called precarthamin (Takahashi et al. 1984), we observed that at room temperature, the flame-coloured pigment gradually changed to reddish orange, a colour similar to that of carthamin. However, the colour change could be restored to some extent by replacing air in the sample storage vessel with argon or nitrogen gas. This suggests the possibility that carthamin also arises from precarthamin through an auto-oxidation reaction during the colour transition of *Carthamus* flowers.

In the present study we investigated the formation of carthamin in bufferized solutions containing precarthamin and oxidizing agents.

MATERIAL AND METHODS

PREPARATION OF PRECARTHAMIN

The method for preparation of precarthamin described previously (Saito et al. 1983a) was slightly modified to purify the compound further. Freshly collected safflower florets (380 g) were homogenized in 1400 cm^3 of cold methanol with a Waring blender for 2 min, and filtered through Toyo Roshi No. 2 paper, washed with more cold methanol and dried over silica gel in a vacuum desiccator. The methanol-dried powder was extracted for 10 min at room temperature with methanol/formic acid (7:3, v/v); the residue, separated by centrifugation, was repeatedly extracted several more times with the same solution. Combined extracts (2900 cm^3) were evaporated *in vacuo* and the dark brown residue was treated successively with Amberlite IR-120B (H^+-form) and Avicel cellulose column as reported previously (Saito et al. 1983b). The resulting reddish yellow precarthamin was purified further by repeating gel filtration on Toyo Pearl HW-40F in methanol/acetic acid/water (6:1:4, v/v) or in methanol/water (65:35, v/v). Flame-coloured crystalline precarthamin (39 mg) was obtained from the final recrystallization process and used in the present study as a substrate.

Whatman CF-11 cellulose powder was obtained from W. & R. Balston Ltd. (London, England). Silica gel and cellulose thin-layer plates were purchased from E. Merck (Darmstadt, West Germany). All chemicals used were of a reagent grade.

COLORIMETRIC ASSAY

The reaction mixture (4 cm^3) contained, if not otherwise mentioned, the following components: 50.0 mM citrate buffer, pH 5.2; 20 μg of precarthamin; 62.5 nmol-312.5 mmol oxidizing agents. In testing the effect of buffers on carthamin formation, 50.0 mM citrate buffer, pH 5.2, was replaced with the same molar concentration of acetate, succinate, citrate-phosphate, phosphate, Tris-maleate, maleate or phthalate, all of which had been adjusted to pH 5.2 prior to incubation. Reactions were started at room temperature (21-22 °C) in a colorimeter (Shimadzu, Type SP-20) immediately after the addition of oxidizing agents to the assay mixture. The synthetic rate of carthamin from the precarthamin was measured by following changes in absorbance at 517 nm during a 5 minute incubation period. The content of carthamin formed was calculated from a standard curve. Antioxidants such as hydroquinone, L-ascorbic acid and D-araboascorbic acid were used for examining the effect on carthamin formation at the concentration of 2μM.

EXTRACTION AND PURIFICATION OF THE REACTION PRODUCT

Purified crystalline powder of precarthamin (2 mg) was added separately to 11 cm^3 of 50.0 mM citrate buffer, pH 5.2, containing 1 g of cellulose powder and 0.4 μmol $KMnO_4$ or 0.8 mmol H_2O_2. The mixture was incubated separately for 20 h at room temperature (17-20°C) by continuous stirring with magnetic stirrers. After removing the supernatant, the pellet was washed several times with distilled water. The reaction product was then extracted exhaustively with 60% aqueous acetone and purified by column chromatographic methods as described previously (Saito et al. 1983b). 1.9 and 0.9 mg of dark reddish masses were obtained at the final purification step of the product by $KMnO_4$ and H_2O_2, respectively. The purified samples were subjected to the chromatographic identification and spectrophotometric analyses which were routinely employed in enzymic studies (Saito et al. 1983a, b).

OXIDATIVE CONVERSION OF PRECARTHAMIN TO CARTHAMIN

Precarthamin (20 μg) was dissolved in 5.0 cm^3 of 50.0 mM citrate buffer, pH 5.2, and the solution was bubbled with oxygen gas (flow rate of the oxygen gas was 3.6 $cm^3 \cdot min^{-1}$) for 20 h at 21°C. After incubation, the carthamin content was determined by measuring its absorption peak at 517 nm.

UV-LIGHT IRRADIATION OF PRECARTHAMIN

A pure sample of precarthamin (20 μg) in 5.0 cm^3 of 50.0 mM citrate buffer, pH 5.2, was irradiated for 20 h at 21°C in a beaker (2.2 × 4.2 cm) with a GL-15 Mercury lamp (20 W). The irradiated liquid was examined in a colorimeter at 517 nm.

RESULTS

SOME CHARACTERISTICS OF PRECARTHAMIN

Precarthamin forms flame-coloured micro-crystals which are slowly decomposed at 150°C. The UV absorption pattern of a purified sample is given in Fig. 1. The spectrum shows maxima at 225 (sh.), 243, 340 (sh.) and 423 nm, typical of a quinoidchalcone glycoside. [1]H-NMR spectral analysis indicated that the pigment has an antisymetric conformation with two equatorially C-1-alkylated glucopyranosides which can be divided into three sub-units such as quinoidchalcone, p-hydroxycinnamic acid and glucose. Detailed conformational assignment of the data from spectral analyses of the precarthamin is undertaken in our laboratories.

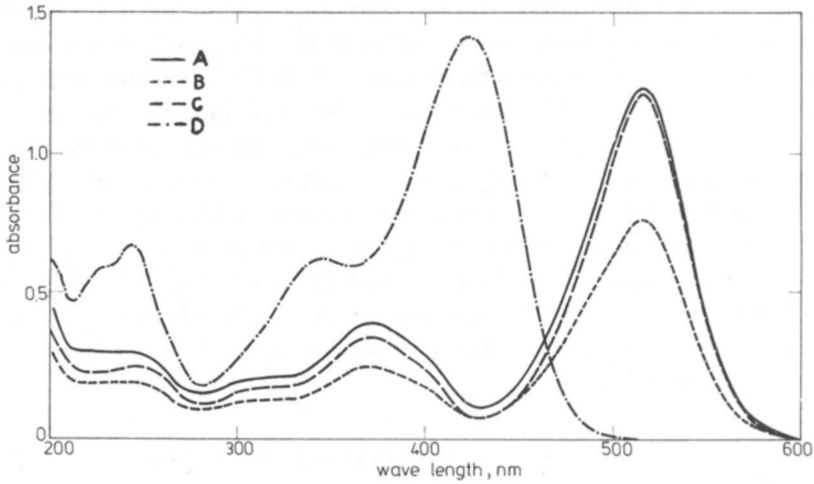

Fig. 1. UV spectra of reaction products, authentic carthamin and precarthamin. The absorption spectrum was recorded with a Shimadzu MPS-2000 spectrophotometer following each addition. The amount of the compound in methanol was ($\mu g \cdot cm^{-3}$): (A) H_2O_2 mediated product, 36; (B) $KMnO_4$ mediated product, 28; (C) authentic carthamin, 50; (D) precarthamin, 20

IDENTIFICATION OF THE REACTION PRODUCT

Standard assays were done using precarthamin and $KMnO_4$ or H_2O_2 as reactants. The purified reaction product was identified by comparing its physical and spectrochemical properties with those of an authentic sample. R_f values of the reddish product in four different solvent systems were almost the same as those of an authentic marker. UV spectra of the almost the same as those of an authentic marker. UV spectra of the reaction products and an authentic specimen in methanol were: 210, 250 (sh.), 310 (sh.), 370 and 517 nm (Fig. 1). The result from IR spectral analysis of the synthetic pigment by $KMnO_4$ gave the same IR absorption pattern as that of an authentic carthamin (Fig. 2). [1]H-NMR spectrum of $KMnO_4$-catalyzed product at 400 MHz in pyridine-d_5/methanol-d_4 proves to be the same spectral pattern reflected by the carthamin structure as reported previously (Takahashi et al. 1982, Obara and Onodera 1979, Takahashi et al. 1984).

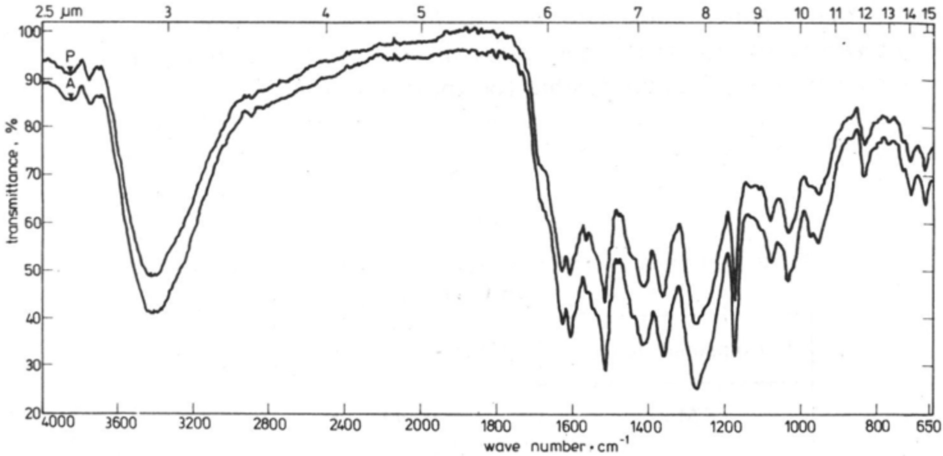

Fig. 2. IR spectra in KBr disks of $KMnO_4$-mediated reaction product and an authentic carthamin. P — $KMnO_4$-mediated product, A — authentic carthamin

STOICHIOMETRY

Precarthamin was incubated with various oxidizing agents in 50.0 mM citrate buffer, pH 5.2, at 21°C. After incubation for 5 min, the reaction product was analyzed. In addition, the change in the absorption rate of the reaction mixture was monitored by a colorimeter just after addition of oxidizing agents to the incubation medium. The content of carthamin produced was estimated from a standard curve as mentioned previously. Results from using three oxidizing agents are summarized in Table 1. Among the oxidizers tested, $KMnO_4$ is potent, while H_2O_2 and HIO_4 are less effective for oxidative conversion of precsthamin to carthamin.

INFUENCE OF pH ON FORMATION OF CARTHAMIN FROM PRECARTHAMIN BY $KMnO_4$

Synthetic activity of carthamin from precarthamin by $KMnO_4$ was found to be optimal between pH 5.0 and 5.6 in 50.0 mM citrate buffer (Fig. 3). At pH 4.2, the activity of carthamin formation was 32% of the optimal value, which is roughly similar to previous findings in enzymic studies (Saito et al. 1983a, b). On the alkaline side at pH 8.0, the synthetic activity was reduced to below 21% of the optimum.

EFFECT OF BUFFER SYSTEMS ON CARTHAMIN FORMATION

Precarthamin was incubated with $KMnO_4$ in various buffers at pH 5.2 instead of citrate buffer which was routinely used in this study. Table 2 shows that citrate, phosphate and acetate are preferable for conversion of precarthamin to carthamin by $KMnO_4$, while succinate, Tris-maleate and maleate are all unfavourable for the reaction.

Table 1

Effect of various oxidizing agents on carthamin formation from precarthamin

Compound tested	Concentration	Carthamin formed, $nmol \cdot min^{-1}$
$KMnO_4$	0.25 μM	2.3
	1.25	7.2
	2.50	32.3
	12.50	295.4
	25.00	297.5
	125.00	153.5
H_2O_2	1.25 mM	3.6
	2.50	31.8
	12.50	36.4
	25.00	36.4
	125.00	64.5
	250.00	55.0
	1250.00	53.0
HIO_4	25.00 mM	3274.8
	125.00	13774.2

50.0 mM citrate buffer, pH 5.2, was used for the incubation medium. The reaction was started immediately after mixing each test compound with the incubation medium at various concentrations as indicated in Table 1. Composition of the reaction mixture and methods for assay were as described in the text.

AUTO-OXIDATION OF PRECARTHAMIN

Precarthamin was oxidized to form carthamin by oxygen gas in a citrate buffer solution. The rate of the auto-oxidation of precarthamin reached 7.3 pmol $carthamin \cdot h^{-1}$ during 20 hours incubation at 21-24°C. UV-light irradiation of precarthamin was also carried out in 50.0 mM citrate buffer, pH 5.2, however, after 20 hours incubation the reaction mixture containing precarthamin was found to be colourless.

DISCUSSION

During the characterization of precarthamin we found that the flame--coloured micro-crystalline powder slowly turns to reddish yellow when it was left open to air or in aqueous solution. This colour change let us wonder if auto-oxidation might be involved in the process of carthamin formation in safflower florets. On the basis of this assumption we examined the non-enzymic synthesis of carthamin under mild conditions as a model for a biological process. Data from the present studies provided us with new information that carthamin could be synthesized from precarthamin through non-enzymic reactions.

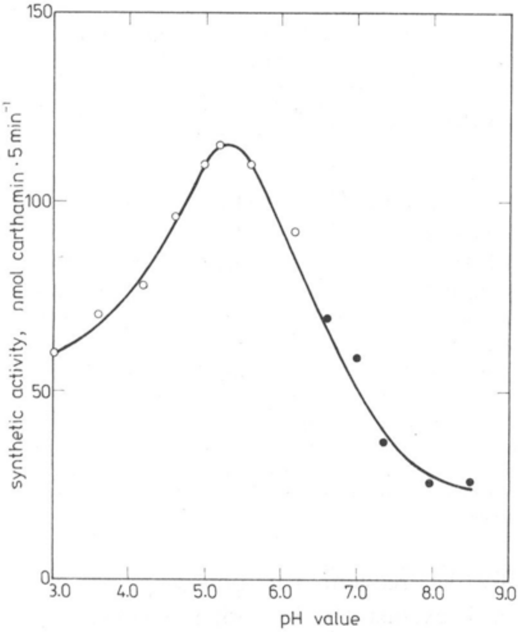

Fig. 3. Effect of pH value on the formation of carthamin from precarthamin by $KMnO_4$. Assay methods were as described in the text except that the buffer used in the incubation medium was varied as shown: (O) 50.0 mM citrate buffer; (●) 50.0 mM Tris-maleate buffer

An oxygen bubbled precarthamin-containing buffer brought a faintly reddish product, which was identified as carthamin by its colouration and chromatographic behaviour. The synthetic rate of carthamin was calculated to be 7.3 pmol carthamin$\cdot h^{-1}$ under the conditions described above. This suggests that auto-oxidation is also possibly cooperative in safflower florets, though the enzyme previously reported (Saito et al. 1983a, b) may play the primary role in carthamin synthesis. In *Carthamus* flowers, light oxidation of precarthamin may also be involved in carthamin synthesis, but no fact could be found in this experiment to support this supposition.

$KMnO_4$, H_2O_2 and HIO_4 were effective for the synthetic production of carthamin from its precursor, precarthamin. Other oxidizing agents such as $K_2Cr_2O_7$, MnO_2, CrO_3, SeO_2 and Ag_2O were found to show little or no effect on carthamin formation. $KMnO_4$ formed 154 nmol carthamin·min^{-1} from precarthamin at the concentration 125 μM, while H_2O_2 and HIO_4 formed 65 and 13774 nmol carthamin·min^{-1} at the concentration of 125 mM, respectively. From the above results $KMnO_4$ can be used

Table 2

Effect of buffer systems on carthamin formation by $KMnO_4$

Buffer system	Carthamin formed, nmol·min^{-1}	Ratio, %
Citrate	442.3	100.0
Citrate-phosphate	426.6	96.5
Phosphate	355.3	80.3
Acetate	393.8	89.0
Succinate	157.4	35.6
Tris-maleate	91.1	20.6
Maleate	23.9	5.4
Phthalate	405.5	91.7

50.0 mM citrate buffer, pH 5.2, was replaced by the same molar concentration of other test buffer solutions, pH 5.2. Synthetic activity of carthamin was assayed for 5 min at 21°C in each test buffer solution containing 20 μg precarthamin and 12.5 μM $KMnO_4$ in a total volume of 5.0 cm^3.

as a far more effective reagent for detecting precarthamin than H_2O_2 which has been used exclusively by other workers for a spray reagent (Shimokoriyama and Hattori 1955). We have already utilized $KMnO_4$ for a colour developer on thin-layer chromatograms throughout the enzymic studies at a concentration ranging from 0.1 to 0.01 mM in aqueous acetone (Saito et al. 1983a, b). $KMnO_4$ reacted most actively with precarthamin at pH 5.3 in citrate buffer. This matches well with that of the enzymic reaction, whose pH optimum was 5.2 in citrate buffer and 5.3 in acetate buffer (Saito et al. 1983a, b). Carthamin formation was greatly affected by buffer systems. Among the buffers tested at pH 5.2, citrate was the most favourable for conversion of precarthamin to carthamin. Citrate--phosphate, phosphate, phthalate and acetate came next. Succinate, Tris--maleate and maleate showed a strong reverse effect on the reaction mediated by $KMnO_4$. It is interesting to note that phosphate does not exhibit any conspicuous inhibition on the carthamin formation, though in biological systems the activity of carthamin-synthesizing enzyme was strongly resticted by phosphate ions (Saito et al. 1983a, b). This indicates that phosphate ions attack an integral part of the enzyme, which reflects crucial depression of the enzyme activity. In this reaction citrate also recovered the inhibition produced by phosphate ions as seen in the enzymic process (Saito et al. 1983a).

Permanganese action was completely hindered by antioxidants. Hydroquinone and ascorbic acid are known to be free radical chain stoppers in oxidative reactions. The mechanism of the inhibitory action of the antioxidants is supposed to be as follows: manganese reacts readily with antioxidants prior to acting on precarthamin to form a free radical(s) which causes a strong restriction of manganese reactivity (Kalus and Filby 1981).

$KMnO_4$ readily synthesizes carthamin from precarthamin in buffer solutions. MnO_4^- may first attack an active site of precarthamin, which triggers electron transfer or intramolecular rearrangement through which, the carthamin structure is formed. However, additional studies would be required to clarify these interesting problems further.

In a biological system we provided evidence that carthamin-synthesizing enzyme was more active under aerobic rather than anaerobic conditions and was greatly inhibited by phosphate ions (Saito et al. 1983a, b). Studies on the mechanism of carthamin synthesis from precarthamin by manganese and carthamin-synthesizing enzyme are under way in our laboratories.

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Badania nad tworzeniem się kartaminy w roztworze buforowym zawierającym prekartaminę i czynniki utleniające

Streszczenie

Badano powstawanie kartaminy w zbuforowanym płynie zawierającym prekartaminę i związki utleniające. $KMnO_4$, H_2O_2 i HJO_4 stymulowały tworzenie się kartaminy. Natomiast $K_2Cr_2O_7$, MnO_2, Ag_2O, SeO_2 i CrO_3 nie wykazały aktywności lub tylko nieznaczną aktywność w katalizowaniu syntezy kartaminy. $KMnO_4$ silnie działało na prekartaminę, podczas gdy H_2O_2 i HJO_4 nie działały. Optymalne pH do tworzenia się kartaminy w obecności $KMnO_4$ wynosiło 5,3 w 50 mM buforze cytrynianowym. Antyoksydanty, takie jak hydrochinon, kwas L-askorbinowy i kwas D-araboaskorbinowy wyraźnie inhibowały oksydacyjną przemianę prekartaminy w kartaminę, w małym stężeniu.