Potential Competency of Glucose Oxidase for Modification of Flower Colour in *Carthamus tinctorius*

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Glucose was found to be a potential stimulator of carthamin formation in triturated florets of *C. tinctorius*. At a 10 mM glucose level, carthamin-producing activity was raised from 3.2-fold (upper) to 2.0-fold (lower) level compared with the control having no sugar. Glucose oxidase (β-D-glucose:oxygen 1-oxidoreductase, EC 1.1.3.4) from *Aspergillus niger* could catalyze the conversion of precarthamin to carthamin, indicative of flower colour modification in *C. tinctorius*. For optimum reaction, precarthamin, β-D-glucose, and oxygen were required. No manganese enhanced the catalytic enzyme process.

Introduction

In recent years, there has been considerable interest in the process of flower colour modification in *C. tinctorius* at *in vivo* and *in vitro* systems [1]. Studies revealed that accumulation of a red pigment in floral parts is responsible for the specific colour change [2, 3]. When a certain pigment content reaches in the florets, the flower colour transition is recognized visually [1]. The chromo-modifying inducer has been characterized to be carthamin, which consists of polyoxy dimeric benzylidene acetophenone structures with C–C glucopyranosyl linkage at 3- and 6-carbon atoms on the mother skeleton [4]. Where the process of its formation was studied, a flame-coloured and oxygen-sensitive intermediate, termed precarthamin, was shown to be an important precursor [5]. Parts of the control mechanism of carthamin formation was clarified indicating that the precarthamin is converted to red carthamin by the action of an enzyme oxidation [2, 6]. Saito *et al.* isolated and partially purified the active fraction which catalyzes the reaction readily [7].

In another series of our experiments with fresh capitula of the garden plant, we have noted that the carthamin-producing activity is promoted markedly by the addition of glucose. Thus, on the basis of our observation, it seems unlikely to rule out the possibility that an additional process is operative leading to the formation of carthamin. The present investigation was undertaken to examine whether glucose oxidase can mediate formation of carthamin from precarthamin in an experimental model system.

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Materials and Methods

Glucose oxidase (β-D-glucose:oxygen 1-oxidoreductase, EC 1.1.3.4) from *Aspergillus niger*, diphenylboric acid-β-ethylamino ester and poly(ethyleneglycol)-4000 were supplied by Wako Pure Chemical (Osaka, Japan). Glucose and fast blue salt B were provided from Kanto Kagaku Yakuhin (Tokyo, Japan). Precarthamin and carthamin from *C. tinctorius* were from our laboratory collection. Silica and cellulose TLC plates were obtained from Merck (Darmstadt, F.R.G.). Avicel cellulose was a product of Asahi Kasei Kogyo (Tokyo, Japan). Toyo Pearl HW-40f was purchased from Toyo Soda Kogyo (Tokyo, Japan). Chromatorex ODS was furnished by Fuji-Davison Chemical (Kasugai, Japan). Other chemicals and reagents used were of analytical grade and purchased commercially.

Seeds of *C. tinctorius* were grown on our experimental field. The plant flowered in July–August, bearing orange-yellow tubular flowers. Bright-yellow flowers were collected from the prematured flower buds and used immediately for the measurement of carthamin-producing activity.

The picked florets (5 g) were ground to be small pieces with a pestle and mortar in liquid nitrogen. To the crushed materials, 20 ml of 10 mM glucose solution was added and left to stand for 30 min at

room temperature. Carthamin formed was extracted and its content determined spectrophotometrically according to the method of Saito [8].

In an experimental model system, glucose oxidase activity was assayed by following the increasing in absorbance at 521 nm with a JASCO double-beam UV/VIS spectrophotometer, model U-best-30 connected with a JASCO CRT-400 monochrome display unit and a JASCO PTL-369S automatic recorder. The standard assay mixture consisted of 0.05 ml of 200 μM precarthamin (calculated in terms of carthamin), 0.1 ml of 556 μM glucose, 0.01 ml of 20.1 munits/ml glucose oxidase, and 2.0 ml of 50 mM citric acid/sodium phosphate buffer, pH 5.5 in a total volume of 2.2 ml. The reaction was started just after addition of enzyme and the mixture was incubated for 2 min at 30 °C in a 1 cm light-path cell, around which warm water was circulated continuously. For obtaining the reaction product, the standard reaction mixture, scaled up to 20 times, was incubated under the standard assay condition, except where the incubation was carried out for 30 min and 500 mg Avicel cellulose was added to the incubation mixture. The reaction was stopped with glacial acetic acid and the mixture was centrifuged for 5 min at 3000 × *g*. The supernatant was discarded and the residue washed five times with sufficient amounts of deionized/distilled water. The red slurry was extracted repeatedly with 60% (v/v) acetone and the pooled extracts were evaporated to dryness at less than 35 °C. The residue was chromatographed successively in *n*-butanol/acetic acid/water (4:1:5, v/v, upper phase), methanol/water (7:3, v/v), and acetone/water (4:6, v/v). Avicel cellulose, Toyo Pearl HW-40f, and Chromatorex ODS were used for the column packings, respectively. The chromatographically purified product (approx. 2 mg) was directly compared with an authentic specimen by one-dimensional TLC on cellulose or silica using at least three of the developing solvents mentioned above. Diagnostic spray reagents used were: diphenylboric acid-β-ethylamino ester/poly(ethyleneglycol)-4000 and fast blue salt B [9]. Plates were occasionally fumed with NH_3 vapour to diagnosticate the coloration [10]. UV spectra were determined with a JASCO U-best-30 spectrophotometer in 70% (v/v) methanol. The sample used for IR-spectral measurement was purified further by a high performance liquid chromatographic method. A Wakosil ODS 5C18 column (5 μm, 20 × 150 mm i.d.) was fitted with a Hitachi L-6200 eluent supply and a Hitachi 1-4200 detector set on 521 nm. Elution with 70% (v/v) acetone (10 ml/min) was used. IR spectra were registered with a JASCO spectrometer, model IR-810 in micro-KBr disks.

Results and Discussion

To justify our previous observation that glucose enhances carthamin-generating activity, we re-examined first its effect with triturated florets (Table I). It is evident from the data that glucose contributes to the pigment production at a visually sizable scale. Upon feeding 10 mM glucose, the carthamin-producing activity is raised up to by 2.6-fold in average comparing with the control to which no sugar was added. Glucose was confirmed to be an effective inducer of carthamin formation. This led us to test glucose oxidase activity in an experimental model containing precarthamin and glucose in an acidic buffer solution. The reaction profile (Fig. 1) shows the interaction between glucose oxidase and precarthamin. On an experimental ground [11] and results from spectrometric (UV and IR; Fig. 1 and 2), and chromatographic evidence (data not shown), it is clear that the single product of the precarthamin applied is identical with carthamin. This is the first evidence that glucose oxidase controls another diversed process of catabolizing a secondary metabolite, precarthamin, which has been little considered yet to serve as an intermediary substrate for the enzymatic catalysis. The findings of this investigation are supported by the fact that glucose promotes the reddening of flower florets from prematured capitula of *C. tinctorius* (Table I). This is substantiated fur-

Table I. Effect of glucose on the reddening of florets from *C. tinctorius*.

Experiment No.	Carthamin formed [nM/fr. wt./min]	Increase[a] [-fold]
I	16.1	1.98
II	25.6	3.14
III	19.8	2.43

[a] Specific value of the control test was 8.14 nM/fr. wt./min. For details of experimental conditions, see Materials and Methods.

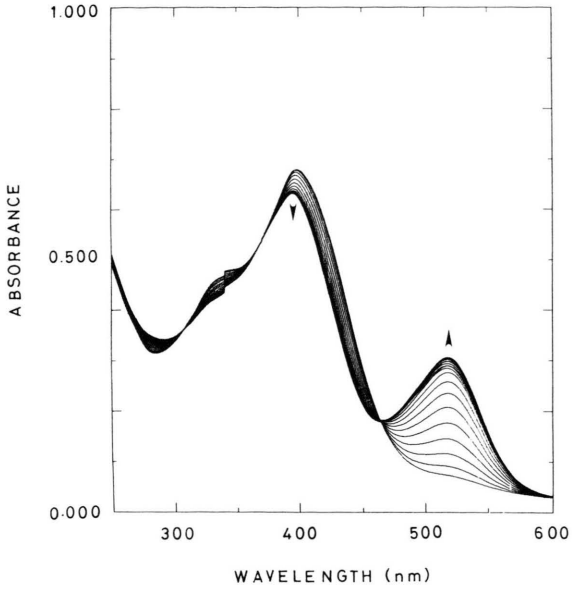

Fig. 1. Light absorption spectra recorded with time intervals of enzyme reaction. The spectra were recorded 20 times at each 5 min interval in a qualz cell containing 0.25 ml of 200 μM precarthamin, 0.1 ml of 556 μM β-D-glucose, 0.1 ml of 20.1 munits/ml of glucose oxidase, and 2 ml of 50 mM citric acid/sodium phosphate buffer, pH 5.5 in a total volume of 2.45 ml. The incubation was carried out at 30 °C. The arrows illustrated in the figure show the direction of the spectral change monitored during the enzyme reaction.

ther by the results from a model experiment, where glucose oxidase catalyzes carthamin formation from precarthamin (see Fig. 1 and 2). In favour of earlier observation [2, 7, 11], the reaction proceeds readily under aerobic conditions (Table II). Apparently, manganese is not to be expectable as positive stimulator of the enzyme activity (Fig. 3).

Table II. Carthamin formation directed by glucose oxidase.

Condition	Carthamin formed [pM/munits/min]	Remaining activity[a] [% of control]
Boiled[b]	0	0
Air removed[c]	19.7	13.8
Glucose omitted	0.74	0.52
Enzyme omitted	0	0

[a] Specific value of the control test was 142.3 pM/munits/min.

[b] Glucose oxidase sample was inactivated by heating at 98 ± 1 °C for 5 min.

[c] Reaction mixtures were pipetted into a Thunberg type cuvette and headspeace air was evacuated removing under reduced pressure. Enzyme activity was assayed immediately after mixing glucose oxidase sample from a side-arm of the cuvette. For further details of experimental conditions, see Materials and Methods.

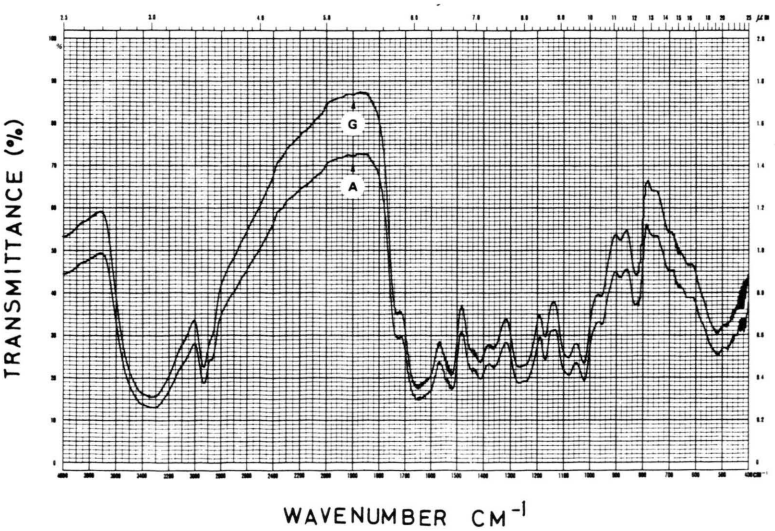

Fig. 2. IR spectra of glucose oxidase-induced product (G) and authentic carthamin (A) recorded with micro-KBr disks.

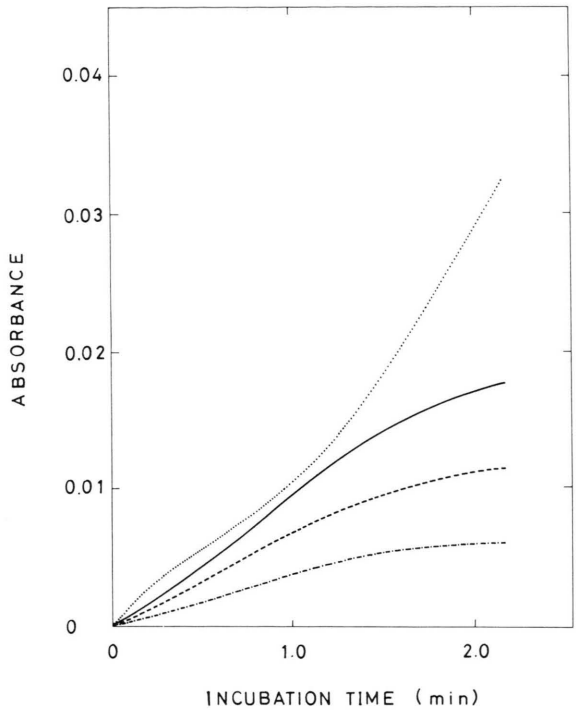

Fig. 3. Effect of divalent manganese on the activity of glucose oxidase and carthamin-synthesizing enzyme. – – – –, glucose oxidase; –·–·–, glucose oxidase + Mn^{2+}; ——, carthamin-synthesizing enzyme; ....., carthamin-synthesizing enzyme + Mn^{2+}. The data of glucose oxidase activity are illustrated emphatically by about four times larger than those which were investigated. Mn^{2+} was fed as acetate salts.

Catalytic conversion of precarthamin to carthamin is also controlled by the action of another enzyme tentatively called carthamin-synthesizing enzyme [2]. Saito *et al.* have declared that the enzyme activity is enhanced considerably in the presence of divalent manganese. Therefore, the unfavourable property of glucose oxidase to manganese ions obviously contrasts with that of carthamin-synthesizing enzyme.

On the assumption that glucose oxidase could take part in carthamin formation, three different processes would possibly be operative in the flower colour transit reaction: two of which are enzymatic, namely glucose oxidase-mediated oxidation of precarthamin and carthamin-synthesizing enzyme-catalyzed formation of carthamin [2], and the third one is non-enzymatic, autooxidation of precarthamin [12].

In *Carthamus* flower colour change, some notable and characteristic aspects seem to be involved especially in tinctorial manifestation and enzyme mechanism. From the beginning of our investigation on transcoloration, we inquired why the activity of the enzyme associated with carthamin formation is distributed widely over vegetative parts of the plant, while flower colour change occurs in limited region of the floral tissues only [2, 6]. The location of the enzyme activity has also puzzled us. Why is the enzyme activity found preferentially in younger and developing parts of the vegetative tissues, whereas the colour modification is manifested at later phase of the blooming stage [1, 2, 6]?

The present data may indicate why carthamin-producing activity spreads over above ground parts of the flowering plant. Apparently, transcoloration reaction in *Carthamus* flowers must be induced *via* accompanied processes controlling specified enzyme mechanisms.

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