

# Acceleration of Enzyme-Dependent Carthamin Formation by Manganese with Diversed Valence States

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Effect of manganese with three different valence states on carthamin formation was studied by using freshly collected flowers from dyer's saffron (*C. tinctorius*) capitula. At 1–10  $\mu\text{M}$  concentration, these cations accelerated markedly the carthamin formation induced by endogenous enzyme(s) in the detached floral tissues. The acceleration was obviously dependent on the valence state of the test metals, more pronounced by Mn(VII) or Mn(III) than by Mn(II) at a series of given ionic strengths. On a 1  $\mu\text{M}$  level, Mn(VII) and Mn(III) accelerated the pigment accumulation by faster of 9 and 8, resp. than Mn(II) during 5 min incubation at 30 °C. Mn(VII) and Mn(III) acted on both fresh and boiled florets, while Mn(II) exerted preferentially its activity on intact materials. Possible implication of manganese ions on the enzyme-catalyzed carthamin formation are discussed.

## Introduction

In some cultivars of dyer's saffron (*C. tinctorius*), we can see that the colour of their capitula changes gradually from orange-yellow to reddish-orange and then to rusty-red. These characteristic transcolourations occur in the floral parts at the later stage of the flowering period under the normal conditions [1, 2].

It has been reported earlier [3, 4] that the bathochromic colour change can be manifested in the presence of proteinaceous extracts and a flame-coloured precarthamin [5] from the herbal plant. During investigation with metal ions, the sensitive nature of the enzyme towards Mn(II) has been suggested [6], where a partially purified preparation from the etiolated seedlings is activated specifically by the addition of the metal cations at 12–600  $\mu\text{M}$  levels.

Oxidative transcolouration is familiar to us. This reaction is known to be controlled by the action of polyphenol oxidase (EC 1.14.18.1) or peroxidase (EC 1.11.1.7). Our previous findings [6–10] strongly indicate, however, that additional new enzyme(s) can also catalyze the transcolouration. It, therefore, seemed to be necessary to study the enzyme property more extensively including metal factors *in vivo* and/or *in vitro* systems.

**Abbreviations:** NP, diphenylboric acid- $\beta$ -ethylamino ester; PEG, poly(ethyleneglycol)-4000.

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This work describes the development of red colour *in vivo* under the influence of Mn(II), Mn(III), or of Mn(VII). From these observation, we will attempt to reveal the possible role of manganese in colour change manifested by endogenous enzyme action in dyer's saffron capitula.

## Materials and Methods

### Materials

Mn(II) acetate and potassium permanganate were from Wako Pure Chemical (Osaka, Japan). Mn(III) acetate was from Aldrich Chemical (Milwaukee, Wis., U.S.A.). Precarthamin and carthamin were from our laboratory collection. Avicel cellulose was obtained from Asahi Kasei Kogyo (Tokyo, Japan), cellulose and silica TLC plates from Merck (Darmstadt, F.R.G.), Sephadex LH-20 from Pharmacia Fine Chemicals (Uppsala, Sweden), Toyo Pearl HW-40f from Toyo Soda Kogyo (Tokyo, Japan), end-capped silica gel (Chromatorex ODS, grade BU 3050 MT) from Fuji-Davison Chemical (Kasugai, Japan). Other chemicals and reagents used were all analytical grade obtainable from several commercial sources. The seeds of dyer's saffron (*C. tinctorius*) were obtained from a market (Yamagata, Japan).

### Cultivation of dyer's saffron

The seeds were selected manually to regularize their germination rates and to ensure their subse-



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quent stable growth. Uniform seeds thus obtained (germination rate 49%) were sown on our experimental field on April 29, 1990. At intervals, water was sprinkled over the field and underdeveloped seedlings were culled out carefully. On July 30, the plants, which were grown up to be erect almost glabrous annual herbs, 30–50 cm tall, bloomed bearing bright-yellow or orange-yellow florets on the urn-shaped terminal heads with numerous spinescent ciliates.

#### *Test for floret reddening by manganese ions*

Capitula (1.5–2.0 cm in diameter) were selected randomly on the field from the flowering heads at the stage of the pre-blooming. A large number of bright-yellow florets (approx. 500 g fresh wt.) was dissected collecting manually from the capitula and stocked immediately in an ice-box. The starting material (4.5 g fresh wt. each) was frozen in liquid nitrogen and crushed into small pieces with a pestle and mortar. Except where indicated otherwise, the resulting fine powders were suspended in 20 ml of 1  $\mu$ M manganese solutions (20 nmol) and the suspension was incubated for 5 min at 30 °C in an open flask (50 ml), which was agitated incessantly at 170 strokes per minutes in a water-bath incubator. Denatured florets were prepared by heating the fresh materials in boiling ethanol for 5–7 min prior to use for the experiments.

In separate experiments to obtain reaction product for analyses, the incubation was performed under the same conditions as described above, except for being scaled up by about 20-fold in the weight of the starting material and the volume of the aqueous solution of Mn(II), Mn(III), or of Mn(VII). When finishing the incubation, all suspensions were heated instantly for 10 min at  $99 \pm 1$  °C and stocked freezing at  $-20$  °C in the dark just before extraction of the incubation product.

#### *Extraction and estimation of incubation product*

The frozen florets were thawed in tap water at  $13 \pm 2$  °C and washed five times with sufficient amount of deionized/distilled water (approx. 300 ml each) on a Büchner funnel. The residue was suspended in 20 ml of 0.5%  $K_2CO_3$  solution, which was stirred vigorously with a magnetic stirrer for 3 min. The extraction was repeated twice with fresh  $K_2CO_3$  solution (20 ml, 0.5%). Solid citric acid

(1 g) was mixed with the combined extracts and the acidic solution was stirred with Avicel cellulose (1 g) for 10 min at 22–24 °C, then it was washed five times with each 200 ml deionized/distilled water on a Büchner funnel by suction. The red Avicel was treated with 50 ml of 60% (by vol.) acetone and the resulting eluate was applied at once to the spectrophotometric estimation. The reading of the light absorption at  $\Delta A \lambda_{Me_2CO}^{max} 521$  nm was monitored with a Hitachi spectrophotometer, model U-1100. The data from the spectroscopic monitoring were compared with a calibration curve and each content of the incubation product in the eluate was computed. The reaction product used for the identification process was isolated and purified through the same techniques as presented in our previous papers [3, 4], except that the product was treated on a Chromatorex ODS column ( $1.3 \times 30$  cm) in acetone/water (7:3, by vol.).

#### *Examination of incubation product*

The red product was examined on cellulose or silica gel TLC plates during isolation and purification steps. Chromatographies were done with the following solvent systems; (A) *n*-butanol/acetic acid/water (4:1:2, by vol.), (B) acetic acid/water (15:85, by vol.), and (C) phenol/acetic acid/water (40:1:10, by vol.). Occasionally, precarthamin was incubated in solutions containing manganese ions or crude cell-free preparations from fresh florets by following the method of Saito *et al.* [3, 6] and resulting red product was co-chromatographed with an authentic carthamin or with the recovered pigment from manganese-treated flowers. Thus prepared air-dried chromatograms were viewed under UV (366 nm) or visible light. NP/PEG [11] and  $NH_3$ -vapour [12] were used as detecting reagents. The chromatographically purified sample was identified unambiguously by direct comparison with an authentic compound. HPLC was carried out using a ODS (Wakosil 5C18) column (5  $\mu$ m,  $4 \times 250$  mm i.d.) fitted with a Hitachi L-6200/L-6000 driving systems and a Hitachi L-4200 detector set on 521 nm. Isocratic elution with 70% (by vol.) methanol (1 ml  $\cdot$  min<sup>-1</sup>) was used. UV/VIS spectra were measured with a Hitachi U-3210 spectrophotometer in 70% (by vol.) methanol. IR spectra were recorded with a Jasco spectrometer, model IR-810 in micro-KBr

disks or often conveniently in an arsenic selenide cell containing 70% (by vol.) methanol and 2.3–610  $\mu\text{g}$  per ml of recovered sample in the range from 650 to 4000  $\text{cm}^{-1}$ .

## Results

### Identification of incubation product

Red extracts from fresh and boiled florets with or without addition of manganese ions were compared chromatographically mainly with their  $R_f$  values and tinctorial characteristics. In all TLC solvent systems (A–C) so far tested, each compound exhibited the same or nearly the same  $R_f$  values. No convincing difference in their colourations was also detected. The red sample from the experiments with Mn(II), Mn(III), and Mn(VII) was co-chromatographed on TLC plates with a standard in developing solvents, A–C. The pigment spot, and run with a marker spot, could obviously be superposed with each other on the same chromatogram examined (cellulose TLC,  $R_f$ s: A = 0.43, B = 0.0, C = 0.29). Repeated CC purification steps led to a preparation with greenish red metallic lustre. Using HPLC with non-linear gradient of aqueous methanol, Mn(VII) induced pigment co-eluted with a marker carthamin at a retention time of 2.28 min. Final proof was obtained when the red sample was analyzed spectroscopically. The IR spectra of the recovered product and a

standard specimen coincided with each other in all details (data not presented).

### Influence of manganese ions on the enzyme-dependent carthamin formation

Contribution of Mn(II) to the red-shift of pre-carthamin solution has already been suggested in our experimental models [6], where enzyme-mediated carthamin formation is enhanced considerably by the cations at 12–600  $\mu\text{M}$  levels. In addition, the red colour shift was also promoted markedly by Mn(VII) in enzyme-free systems [13]. In order to confirm the direct participation of the metal ions to intact floret reddening, three Mn(II), Mn(III), and Mn(VII) were tested at various concentrations.

(A) *Valence state and ionic strength.* Table I presents results from the test of manganese with different valence states at various concentrations. Each type of manganese ions increases the rate of carthamin formation, where individual value is exhibited as carthamin content in a net volume of the test solution. Among these cations, Mn(VII) and Mn(III) markedly enhance the enzyme-dependent carthamin accumulation. After incubation for 5 min at 20 nmol level, they increased by faster of 10.4 and 8.1 compared with no manganese-added control. Mn(II) had far lower impact on the enzyme than the other two. This increase in the

Table I. Effect of manganese ions on carthamin formation in detached dyer's saffron florets.

Manganese	Concentration [mM]	Carthamin formed [ $\mu\text{M}$ ]	Specific value [ $\mu\text{M}$ carth · mM Mn <sup>-1</sup> · g flor <sup>-1</sup> · min <sup>-1</sup> ]
Mn(II)	0.001	2.25	100.0
	0.01	7.92	35.2
	0.1	9.24	4.11
	1	12.97	0.58
	10	15.16	0.067
Mn(III)	0.001	18.67	829.8
	0.01	16.21	72.0
	0.1	12.03	5.36
	1	10.24	0.46
	10	2.31	0.011
Mn(VII)	0.001	23.73	878.9
	0.01	16.56	73.6
	0.1	17.21	7.65
	1	18.80	0.83
	10	0	0

carthamin productivity was reduced with preceding manganese ion amplification.

(B) *Incubation time.* In a series of experiments performed under the conditions identical with those for experiments in Table I, except where manganese ions were used at 20 nmol level as indicated in the parenthesis of Fig. 1. For the no ion-added control, the values of all variables with manganese increase fairly the carthamin productivity during the course of the incubation. These suggest that the manganese-stimulated pigment formation is strictly time-dependent. Here, the in-

clination of native enzyme activity for Mn(VII) also overcomes other two cations, Mn(II) and Mn(III).

(C) *Effect of manganese ions on boiled florets.* We performed further experiments to demonstrate directly the participation behaviour of manganese in the carthamin production (Fig. 2). It is similar experiments as described in Fig. 1, except for omitting enzyme activity by heat denaturation. With respect to the carthamin productivity, Mn(III) and Mn(VII) are both potent stimulators, although their effects are by far lower than those of being observed with fresh materials. Mn(II) essentially does not affect carthamin productivity in boiled tissues.

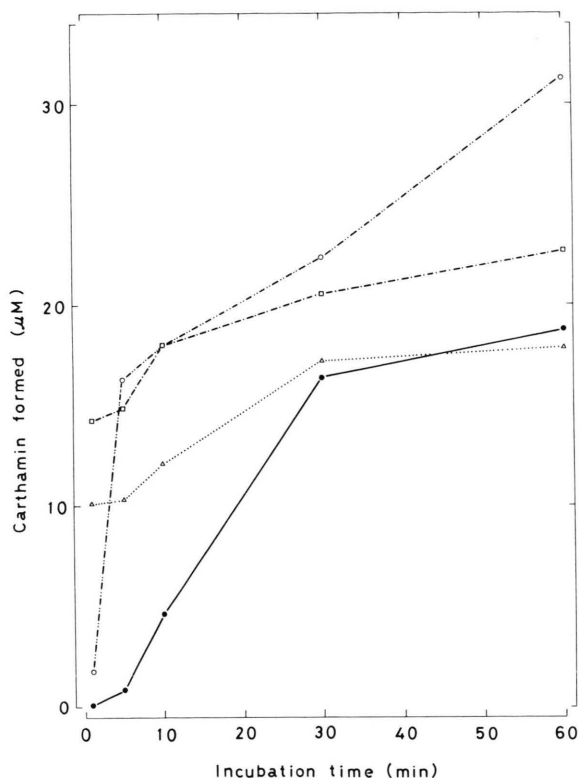


Fig. 1. Time dependence of carthamin formation in fresh florets in the presence of manganese ions. Fresh florets (4.5 g fresh wt. each) were suspended in deionized/distilled water (20 ml) containing each 20 nmol Mn(II), Mn(III), and Mn(VII). Tests for the control were carried out with fresh florets (4.5 g fresh wt. each) and deionized/distilled water (20 ml each) without addition of manganese ions. Individual suspension was incubated for fixed intervals as indicated in the figure at 30 °C by agitating vigorously in a water-bath incubator at 170 strokes per min. Further details for extraction and estimation of the incubation product, see Materials and Methods. Mn(II):  $\triangle$ — $\triangle$ , Mn(III):  $\square$ — $\square$ , Mn(VII):  $\circ$ — $\circ$ , control:  $\bullet$ — $\bullet$ .

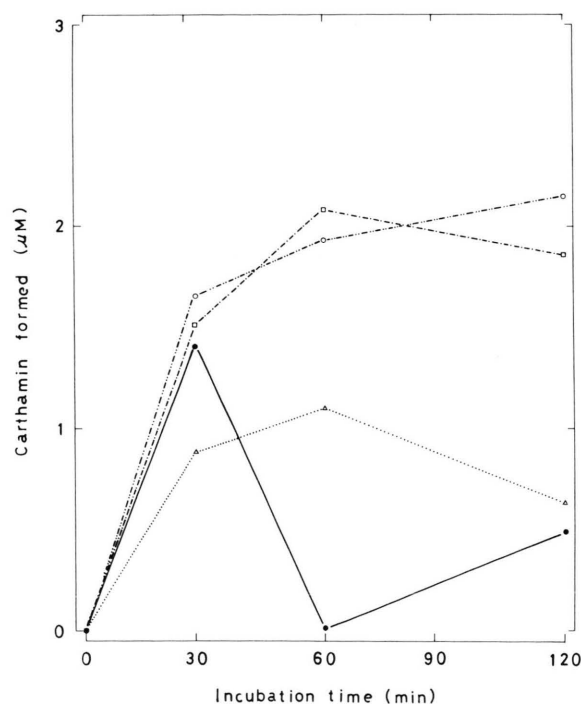


Fig. 2. Time dependence of carthamin formation in denatured florets in the presence of manganese ions. The conditions for incubation of florets were the same as described in Fig. 1, except that denatured florets, which were prepared by boiling the fresh florets (4.5 g fresh wt. each) in ethanol, were used. For further details of the experimental conditions, see Materials and Methods. Mn(II):  $\triangle$ — $\triangle$ , Mn(III):  $\square$ — $\square$ , Mn(VII):  $\circ$ — $\circ$ , control:  $\bullet$ — $\bullet$ .



## Discussion

The current studies on the manganese-induced carthamin formation in detached dyer's saffron flowers have revealed some notable features with regard to the floret colour manifestation pattern. The data indicate that there are plural differences in the reaction induced *in vivo*: (a) manganese interacts with enzyme(s) medially and (b) it reacts with precarthamin directly. Consequently, enzyme-catalyzed pigment accumulation is accelerated strikingly by the presence of the metal cations. This stimulation correlates well with the valence states of the test metals (see Table I and Figs 1 and 2), showing an explicit order: Mn(VII) > Mn(III) > Mn(II). Both Mn(VII) and Mn(III) enhanced the red colour manifestation, even whose enzyme(s) had been denatured previously by heating. While only suspectable activity was found in Mn(II) with boiled materials, which is a marked contrast to the pigment productivity of Mn(VII) or of Mn(III). The specific value of manganese concentration *vs.* pigment content (Table I) shows that the enzyme-mediated pigment production depends obviously on the ionic strengths. The pigment productivity decreased greatly at higher ion levels (see Table I). When the ionic strength was multiplied by ten, the value diminished by 72–94% at the higher concentration. Besides, with the excess ions, a brown precipitate, perhaps, manganese hydroxide formed within a few minutes, especially in case of Mn(VII) being used. The rapid precipitation of the hydroxide probably accounts for lowering the pigment productivity in the reaction medium observed at higher ion levels during the course of the incubation.

As well known, some of the oxidative reactions, including polyphenol peroxidation [14–19], seem to require multivalent manganese with the cations acting as super-acid catalysis within the active site of the enzymes. Mn(III) generally acts as strong pro-oxidant, which is reduced by water and undergoes

disproportionation reaction:  $2\text{Mn}^{3+} + 2\text{H}_2\text{O} \rightarrow \text{Mn}^{2+} + \text{MnO}_3^{2-} + 4\text{H}^+$  [20]. The highest state of manganese, Mn(VII) is well known for its uses as a potent oxidizing agent [13]. It reacts in neutral to weakly acidic solutions as follows:  $\text{MnO}_4^- + 4\text{H}^+ + 3\text{e}^- \rightarrow \text{MnO}_2 + 2\text{H}_2\text{O}$ . Thus, above test metals must play an integral role in enzyme-dependent carthamin accumulation through switching their redox potentials.

We have observed that Mn(VII) and Mn(III) stimulate positively the red colour manifestation both in fresh florets and boiled ones, whereas Mn(II) is potent solely in fresh materials. It seems likely that Mn(VII) and Mn(III) have almost similarly greater activity than Mn(II) in the active site of the enzyme.

From the present data, there is a variety of modes that could predict the activation behaviour of manganese with precarthamin-oxidizing enzyme(s). However, it is impossible to elucidate the function of the metal ions at any of the catalytic sides. Characterization of the manganese ion requirement of enzyme may therefore prove useful in examining the mechanism of manganese in enzyme action.

This study is the first report to demonstrate the direct participation of multivalent manganese on the flower colour transition. No investigation has yet been done on the topics, although this attempt seems to be indispensable to reveal the metal co-factor roles in the catalytic enzyme mechanism. In this work we corroborated firmly that the trans-colouration is stimulated not only by Mn(II), but also by Mn(III) and Mn(VII). It is indicative from this observation that flower colour modification is induced by unique enzyme(s) or enzyme system(s) that has never hitherto been characterized in this herbal plant. Further works will seek to distinguish the role of each manganese cations which may serve in the catalytic centre of the enzyme as structural and functional or as modifiers.

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