Effect of pyridine nucleotides on the catalytic activity of a carthamin-synthesizing enzyme from dyer’s saffron (Carthamus tinctorius L.) seedlings

K. Saito
Department of Bioscience and Technology, School of Engineering, Hokkaido Tokai University, Sapporo 000-0821, Japan

Received 22 September 1997; revised 6 January 1998

The effect of NADPH, NADP⁺, NADH and NAD⁺ on carthamin formation was investigated with a partially purified enzyme extract from dyer’s saffron (Carthamus tinctorius L.) seedlings. In the presence of 10 pmol Mn²⁺, 10 pmol NADPH enhanced carthamin formation, catalyzed by a carthamin-synthesizing enzyme most prominently (13.8% increase). In the presence of Mn²⁺, NADH gave the second best result (7.2% increase). The promotive carthamin formation activities of NADPH and NADH were strongly dependent upon the presence of Mn²⁺. Under the same experimental conditions, neither NADP⁺ (2.9%) nor NAD⁺ (4.0%) showed such positive effects as did NADPH or NADH. A conceivable participation of pyridine nucleotides on the catalytic activity of a still unnamed carthamin-synthesizing enzyme is proposed in view of the experimental findings.

Keywords: Carthamin formation, carthamin-synthesizing enzyme, pyridine nucleotide, dyer’s saffron (Carthamus tinctorius L.) seedling.

Introduction
Carthamin is known to be formed from a precursor, pre-carthamin, via enzymatic (Saito et al. 1983; Saito 1992) and non-enzymatic processes (Saito & Takahashi 1985; Saito & Utsumi 1996), the former of which can be further subdivided into two catalytic branches, namely the direct (Saito et al. 1983; 1985) and indirect (Saito 1992; 1993) enzymatic branches. The direct control of carthamin formation is initiated by an enzyme preparation of dyer’s saffron (Carthamus tinctorius L.) tentatively named carthamin-synthesizing enzyme (Saito et al. 1983). The enzyme requires O₂ for the reaction to proceed and is also dependent on the presence of Mn²⁺. To the contrary, other metal cations such as Fe²⁺, Fe³⁺ and Cu²⁺ as well as phosphorus substances inhibit enzyme activity (Saito et al. 1985). These facts led us to believe that the carthamin-synthesizing enzyme differs from ferruginous peroxidase and coppery polyphenolase. Indeed, no carthamin-in-synthesizing reaction in the presence of guaiacol or 3,4-dihydroxyphenylalanine is catalyzed by the enzyme (Homma et al. 1985 and unpublished results). Although a co-factor requirement for the enzyme action has long been expected, no studies have been carried out to date.

In this study, we tested the effect of four co-factors, namely NADPH, NADP⁺, NADH and NAD⁺, on the carthamin-synthesizing enzyme activity. The main aim was to obtain more information on the enzyme in order to distinguish its properties more clearly from those of peroxidase and polyphenolase. It was also envisaged that this could lead to insight into the enzyme mechanism of carthamin formation from precarthamin in dyer’s saffron tissues.

Materials and Methods
The precarthamin sample was from our laboratory stock. NADPH, NADP⁺, NADH and NAD⁺ were purchased from Wako Pure Chemical (Osaka, Japan). Sephadex G-25 and G-100 were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Plant materials were prepared from seeds of Mogami-Benibana, a cultivar of dyer’s saffron (Carthamus tinctorius L.), which were sown on damp Vermiculite and cultured at 23 ± 2°C in the light for 2 weeks.

Preparation of carthamin-synthesizing enzyme extract
Green seedlings (14.3 g fresh weight) were dipped in 70% (v/v) ethanol for 3 min to sterilize the plant surface, then washed 4-5 times with sufficient amounts of distilled water until the smell of alcohol could no longer be detected. Seedlings were macerated in 50 mM citrate/phosphate buffer, pH 7.0, containing 0.1 mM 2-mercaptoethanol and 20 mM D-iso-ascorbic acid, and squeezed through a nylon cloth. The filtrate was centrifuged at 13000 x g for 20 min. The supernatant was fractionated with solid (NH₄)₂SO₄ and the fraction precipitated between 0-80% (NH₄)₂SO₄ saturation which was collected by means of centrifugation (13000 x g, 5 min). Resalting of the protein pellet was obtained by dissolving it in 50 mM citrate/phosphate buffer, pH 7.0, applying it onto a Sephadex G-25 column (2.5 x 20 cm), equilibrated and eluted with the same buffer. The column eluate was fractionated again with (NH₄)₂SO₄ and the protein which precipitated between 0-80% saturation was dissolved in 2 ml of 50 mM citrate/phosphate buffer, pH 7.0. The protein solution was transferred to a Sephadex G-100 column (0.9 x 90 cm) and developed with 50 mM citrate/phosphate buffer, pH 7.0. Fractions of 4.4 ml were collected at a flow rate of 0.12 ml/min and retained at 3–4°C until the following enzyme assay procedure could be carried out.

Enzyme assay and activity determination
The standard reaction mixtures contained, if not stated otherwise, 3 ml of 50 mM acetate buffer, pH 4.8; 0.1 ml protein solution (8.5–9.4 pg protein), 0.116 μmol pre-carthamin. The mixtures were incubated at 30°C for 5 min. Enzyme activity was monitored automatically with a Hitachi, model U-3210 spectrophotometer. The data from the spectrophotometer readings at A₁,0₂ were used for the enzyme activity determination. The blank test was carried out under the same conditions as described above, except that no precarthamin was added to the reaction mixtures. Protein contents were estimated by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Results and Discussion
Evidence is presented here for the influence of pyridine nucleotides on the carthamin formation in vitro. On incubating protein extracts from green seedlings of dyer’s saffron together with precarthamin under the standard assay condition, a considerable amount of carthamin was formed. In the presence of 10 pmol Mn²⁺, both NADPH and NADH enhance the enzyme activity.
promisingly. NADPH was more effective (13.8% increase) than NADH (7.2% increase) (Figure 1). The presence of both NADP+ (2.9%) and NAD+ (4.0%) tended to increase enzyme activity but not so effectively than either NADPH or NADH. When Mn2+ was omitted, carthamin production was reduced and the effect of NADPH and NADH was clearly diminished (Figure 2).

Direct participation of the carthamin-synthesizing enzyme in carthamin synthesis has already been reported in our previous work (Saito et al. 1983). Even with no addition of co-factors of the reaction system, the catalytic protein induces carthamin formation from precarthamin. Yet externally supplied Mn2+ stimulated the enzyme action (Saito et al. 1985; Saito 1991 and Figure 1). It is assumed that Mn2+ may associate closely with co-factor effects on the carthamin-synthesizing enzyme. Indeed, a high productivity of carthamin was induced by both NADPH or NADH in the presence of Mn2+.

It is postulated that carthamin production by the carthamin-synthesizing enzyme is controlled by electron chain reactions. If this assumption can be proved, in future it may indicate that the carthamin-synthesizing enzyme is an oxidoreductase acting on paired donors with incorporation of molecular oxygen (Dixon & Webb 1967). However, final conclusions regarding this enzyme property will only be drawn by using highly purified enzyme preparation. Unfortunately its purification is difficult to achieve as the catalytic protein is very unstable under normal experimental conditions. Moreover, enzyme extracts are sensitive to oxygen and freezing reduces its activity. This latter problem will have to be sorted out first before any of the assumptions made above can be verified.