THE SENSITIVITY OF CARTHAMIN AND SAFFLOR YELLOW B COLOURS TOWARDS REDOX SUBSTANCES

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ABSTRACT

The sensitivities of carthamin and safflor yellow B towards oxidants and antioxidants were compared in bufferized solutions at various given concentrations. The test chemicals affected variously on the pigment colourations: (1) externally charged O_2 had less effect on the colour change of carthamin, (2) orange-yellow coloured safflor yellow B was bleached by the gas flush, (3) carthamin showed little or no affinitive property for H_2O_2 at 0.001-230 μM level, (4) safflor yellow B reacted positively with the oxidant and was changed readily to faint yellow unknown compounds, (5) double-faced responses could be found in carthamin solution with L-ascorbic acid or with D-isoascorbic acid, namely the former antioxidant intensified the colour of carthamin and the latter reduced the red colouration, (6) hydroquinone emphasized the red colour of carthamin in solution, (7) safflor yellow B shifted bathochromically in hydroquinone containing solution, however, it showed no shift in L-ascorbic acid and D-isoascorbic acid solutions. The data are discussed from the standpoint of oxidative dissimilation of the quinochalconoids in the floral tissues of dyer's saffron capitula.

KEY WORDS: carthamin, safflor yellow B, sensitivity, redox substances, dyer's saffron (*C. tinctorius*).

INTRODUCTION

Numerous reports on the oxidative catabolism of flavonoids has now been accumulated in the literature, in which evidences have been shown, indicating that flavones, flavonols, isoflavones, chalcones, aurones and other related compounds can be dissimilated readily (Noguchi and Mori 1969, Wong and Wilson 1976, Barz and Wiermann 1981). The mechanisms of their catalytic turnover processes are also revealed partially both *in vivo* and *in vitro*, occasionally by using radio-active isotopes and/or regulatory proteins (Barz 1981). Thus, our basic knowledge about their dynamic states in living systems seems to be roughly established. However, in spite of devoted and laborious efforts of many contributors, the evidences hitherto submitted are still too poor to interpret whole mechanisms of the flavonoid turnover processes, because more and more occurrence of flavonoids and related compounds with further complicated and unique structures has increasingly been detected in the vascular plant tissues (Rózsa et al. 1981, Camarda et al. 1981, Szilagyí et al. 1981, Kéry et al. 1985, Onodera et al. 1989).

Carthamin and safflor yellow B are both *Carthamus* plant pigments which have been shown to be constructed by synonymous dimeric chalconoid structures (Fig. 1a and b) (Takahashi et al. 1982, 1984). They are, as biological metabolites frequently are, very unstable and easily convertible to unknown substances with hypsochromically modulated various colours. In the floral tissues of dyer's saffron, this dynamic change is apparently intensified particularly at the pre-senescent stage of the inflorescence, where certain internal metabolic disturbance is introduced perhaps through drastic regulatory change in the physiological process (Saito

Fig. 1. Structures of carthamin (a) and safflor yellow B (b)

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1989). Supporting this, some indications have already been presented: sugars promote reddening of the triturated florets (Saito 1992a), glucose oxidase regulates the reaction (Saito 1992b, Saito and Katsukura 1992a), H_2O_2 is an essential factor for the catalytic enzyme mechanism (Saito 1992c).

In a series of our recent investigations on the catabolism of these unique glycosides in *Carthamus* flowers, we have found that carthamin is bleached by peroxidative enzyme oxidation (Kanehira and Saito 1990). Safflor yellow B is also convertible to other compounds by the action of an oxygenative enzyme which differs from peroxidases by its heterogenous property (Saito and Katsukura 1992b). During the enzymic investigation, we have noticed that some notable aspects are seen in the sensitivities of carthamin and safflor yellow B towards certain oxidants and antioxidants. The preliminary evidence prompted us to examine the properties more precisely. Through the systematic investigations, some insight into the characteristic mechanism of the chalconoid dissimilating enzymes could be gained practically.

Comparison of the sensitive properties of carthamin and safflor yellow B towards certain chemicals retaining redox activities is the main object of the present investigations.

MATERIALS AND METHODS

CHEMICALS

Carthamin and safflor yellow B used were prepared from fresh florets of dyer's saffron capitula following the methods of Saito et al. (1989a) and Saito (1991). The analytical grade of H_2O_2 , NaH_2PO_4 , Na_2HPO_4 , L-ascorbic acid, hydroquinone, sodium citrate, citric acid and glycine were purchased from Wako Pure Chemical (Osaka, Japan). Tris was obtained from Sigma Chemical (St. Louis, MO, USA). D-Isoascorbic acid was supplied from Nakarai Tesque (Kyoto, Japan). Other chemicals used throughout this investigation were furnished from several commercial sources.

PREPARATION OF TEST REACTANTS AND BUFFERS

The test reactants and buffers used were prepared at least one- or two-day before carrying out the experiments to ensure the complete solubilization of compounds to be used. A given amount of carthamin or safflor yellow B was dissolved into deionized water and kept in the dark in a cooler at $5 \pm 1^\circ C$ just before use. Two types of 50 μM buffers, if not stated otherwise, citric acid/sodium dibasic phosphate, pH 6.2 and Tris/HCl, pH 8.0 were prepared and stocked at $5 \pm 1^\circ C$.

COMPARISON OF THE SENSITIVITY OF CARTHAMIN AND SAFFLOR YELLOW B TOWARDS THE REDOX CHEMICALS

An aliquot of the solution of carthamin or safflor yellow B was added separately to a quartz cuvette, which had previously been filled with desired buffers and test chemicals. The contents were stirred with a glass rod. After 1 minute's pre-incubation, the spectrophotometric measurements were carried out at $22^\circ C$ for 2 min by monitoring the absorbance change at ΔA 521 nm (carthamin) or of ΔA 411 nm (safflor yellow B). The data from the spectroscopic readings were

used for calculation of the contents through consulting with the calibration curves prepared by using authentic specimens. The repeated monitoring of the spectral change was recorded automatically at desired time intervals over a wide absorption range from 250 to 600 nm.

INSTRUMENTS USED FOR THE SPECTROSCOPIC MEASUREMENTS

A Hitachi UV/VIS spectrophotometric apparatus, model U-3210 was used exclusively for monitoring the settled absorption peaks or of the repeated recording of long range absorption spectra. The cuvette holders were maintained at a stationary controlled temperature with warm-water surroundings. Supplementarily, a Hitachi spectrophotometer, model U-1100, was also used for the reading of the settled wavelength changes.

Fig. 2. Optimum pH of reaction velocity in carthamin solution (a) or in safflor yellow B solution (b).

Each reaction was performed under the standard assay conditions but with buffers and pH substitutions as indicated. Buffers used at 50 mM concentration were: citric acid/sodium dibasic phosphate (●), sodium monobasic phosphate/sodium dibasic phosphate (▲), Tris/HCl (○) and glycine/NaOH (□). For further details, see Materials and Methods. Velocity units are $\mu mol \cdot min^{-1}$.

Abbreviations:

Tris - tris (hydroxymethyl) aminomethane,
 SDS - sodium dodecyl sulphate,
 UV - ultraviolet light,
 VIS - visible light

Fig. 3. Time scanning of the reaction induced in carthamin solution. Absorption spectra were recorded at the time intervals (10 min) at 22 °C. The reaction mixture contained 100 n mol carthamin and 50 mM citric acid/sodium dibasic phosphate buffer, pH 6.2 in a total volume of 3.0 cm³: (a) buffer without O₂ bubbling, (b) buffer with O₂ bubbling for 10 min at 22 °C, (c) buffer with 15 μM H₂O₂. The arrows show the direction of absorbance change induced during the course of the incubation

RESULTS

Based on our previous observations (Kanehira and Saito 1990, Saito and Katsukura 1992b), we examined at first the reaction velocity of the colour changes in carthamin and safflor yellow B solutions. Here, four different buffer systems were tested at the same ionic strength but their pH values adjusted variously. As illustrated in Fig. 2a and b, the pH optima are 6.2 for carthamin and 8.0 for safflor yellow B, respectively. These data fit well with those of our previous observations (Kanehira and Saito 1990, Saito and Katsukura 1992b).

The sensitivities of the quinoidchalcones towards atmospheric O₂ were investigated at various time intervals. Typical results are given in Figs. 3a and 4a,b. It is obvious from the data that dissolved O₂ affects variously the colouration of both carthamin and safflor yellow B. In general, at constant level of the pigment contents, O₂ is apt to intensify the red colour of carthamin in solution progressively at first 20-30 min, indicating that its effect is time-dependent. Then the colour is reduced gradually, because of its feeble nature in aqueous solutions (Saito and Fukushima 1986). With safflor yellow B, O₂ reduces its characteristic orange-yellow colour to a certain hypsochromic region (from 411 to 282-284 nm). The above exhibited contrasts are summarized in Figs. 5 and 6: a main band at 521 nm carthamin raises progress-

Fig. 4. Time scanning of the reaction induced in safflor yellow B solution. Absorption changes were monitored at the time intervals (10 or 20 min) at 22 °C. The reaction mixture contained 100 nmol safflor yellow B and 50 mM Tris/HCL buffer, pH 8.0 in total volume as follows (cm³): (a) buffer without O₂ bubbling (3.5), (b) buffer with O₂ bubbling for 10 min at 22 °C (3.5), (c) buffer with 15 μM H₂O₂ (3.65). The arrows show the direction of absorbance change induced during the course of the incubation

Fig. 5. Time dependence of the reaction induced in carthamin solution. The spectral changes were recorded at ΔA 521 nm at the time intervals as indicated in the figure. For details of the mixture compositions and assay conditions, see Fig.3 and Materials and Methods

ively at first 20-30 min, then drops gradually during further prolonged incubation (Fig. 5), the absorbance peak at 411 nm of safflor yellow B decreases constantly with duration of the incubation time (Fig. 6). The reaction velocities of the chemical shifts observed were $56.3 \text{ nmol} \cdot \text{min}^{-1}$ for carthamin (bathochromic=hypsochromic) and $6.77 \text{ nmol} \cdot \text{min}^{-1}$ for safflor yellow B (hypsochromic). The tests with O_2 flush are also presented in Figs. 5 and 6. Both results support the above findings that O_2 participates directly in the colour shift of the two chalcoquinoid glycosides, although what mechanism by which an initial bathochromic colour change is induced in

Fig. 6. Time dependence of the reaction induced in safflor yellow B solution. The spectral changes were recorded at ΔA 411 nm at the intervals as indicated in the figure. For details of the mixture compositions and assay conditions, see Fig.4 and Materials and Methods

Fig. 7. Effect of H_2O_2 concentration on carthamin oxidation. The reaction mixture (3.0^3) contained: 50 mM citric acid/sodium dibasic phosphate buffer, pH 6.2; 100 μM carthamin; various H_2O_2 concentrations (0.001-200 μM) as indicated. The spectrophotometric assays were performed for 2 min at 22°C after addition of H_2O_2 to the reaction system

carthamin solution remains unknown at present. The observed reaction velocities of the chemical shifts were $56.3 \text{ nmol} \cdot \text{min}^{-1}$ for carthamin (bathochromic=hypsochromic) and $6.59 \text{ nmol} \cdot \text{min}^{-1}$ for safflor yellow B (hypsochromic).

H_2O_2 reacts with the test pigments in somewhat characteristic manners. At 0.01-230 μM level, the oxidizing agent does not exhibit its clear activity on carthamin, while it reacts positively with safflor yellow B and converts readily to certain faint-yellow components (Figs 7 and 8). The bleaching activity of safflor yellow B by H_2O_2 is accentuated linearly up to about 30 μM , then it decreases progressively and reaches a

Fig. 8. Effect of H_2O_2 concentration on safflor yellow B oxidation. The reaction mixture (3.7 cm^3) contained: 50 mM Tris/HCL buffer, pH 8.0; 10 μM safflor yellow B; various H_2O_2 concentration (0.5-230 μM) as indicated. The spectrophotometric assays were carried out for 2 min at 22°C . The inset in the figure shows a double reciprocal form of the reaction velocity versus H_2O_2 concentration

constant level at about 80 μM H_2O_2 . The value of 58.5 μM H_2O_2 for safflor yellow B was obtained from double-reciprocal plots of the H_2O_2 saturation curve of Fig. 8 (see the inset in Fig. 8). The time-course of the incubation from individual experiment with carthamin H_2O_2 or with safflor yellow B- H_2O_2 system is added in Figs 3c and 4c. Marked contrasts in the repeated spectroscopic drawings display the type of the reaction course of carthamin and safflor yellow B towards the fed H_2O_2 at a given concentration. Only little response change appears in the chart of the reaction proceeded in carthamin- H_2O_2 solution [see Fig. 5, the reaction velocity of the chemical shifts (bathochromic=hypsochromic) observed was 59.1 $\text{nmol}\cdot\text{min}^{-1}$, supporting the result of Fig. 3c]. It is clear, from the intermittent monitoring data (Fig. 4c) provided during the reaction course, that H_2O_2 affects the safflor yellow B colouration to reduce its original absorption maximum at 411 nm and increase alternatively a new spectral band at 282-284 nm accompanying a distinct hypsochromic shift at 315-330 nm. The rate of the spectral change, that taken at time intervals of 30 s in the midst of the H_2O_2 catalysis, varies during the incubation, showing that the initial change in optical density at 411 nm follows first-order reaction kinetics [see Fig. 6, the reaction velocity of the chemical shift (hypsochromic) observed was 12.78 $\text{nmol}\cdot\text{min}^{-1}$].

The emphasized differences in the sensitive natures of carthamin and safflor yellow B towards other chemicals were also pronounced by exposing these pigments to antioxidants. Three typical antioxidants, L-ascorbic acid, D-isoascorbic acid hydroquinone were used in this study at various concentrations. The results are given in Figs 9 and 10. Both L-ascorbic acid and hydroquinone intensify the red colouration of carthamin, while D-isoascorbic acid reduces the colour of the pigment (Fig. 9). Safflor yellow B seems to be inert to L-ascorbic and D-isoascorbic acids. Hydroquinone increases the absorption maximum at 411 nm of safflor yellow B (see Fig. 10).

Fig. 9. Influence of antioxidants on the oxidative dissimulation of carthamin. The reaction mixture (3.0 cm^3) contained: 50 mM citric acid/sodium dibasic phosphate buffer, pH 6.2; 100 μM carthamin; various concentrations of antioxidants as indicated. The incubation was carried out for 2 min at 22 °C and the spectral change at ΔA 521 nm monitored automatically. For further details of the experiments, see Materials and Methods

Fig. 10. Influence of antioxidants on the oxidative dissimulation of safflor yellow B. The reaction mixture (3.7 cm^3) contained: 50 mM Tris/HCL buffer pH 8.0; 20 μM safflor yellow B; various concentrations of antioxidants as indicated. The incubation was carried out for 2 min at 22 °C and the spectral change at ΔA 411 nm monitored automatically. For further details of the experiments, see Materials and Methods. —○—○—, L-ascorbic acid; —●—●—, D-isoascorbic acid; —△—△—, hydroquinone

DISCUSSION

The current studies on the sensitivities of quinoidchalcone glycosides towards certain oxidants and antioxidants indicate clearly that these pigments react with the fed chemicals in a somewhat selective manner: initially, the red colour of carthamin is intensified in aqueous solutions followed by a gradual decrease, safflor yellow B incessantly fades away in solutions. Carthamin resists the electrophilic attack of oxidants. Safflor yellow B, on the contrary, is very sensitive to oxidants and readily convertible to hypsochromically coloured compounds. The reason why the chemical response of the chalconoids is varied according to the externally fed substances as yet remains uncertain.

Structurally, the two pigments are composed of synonymous mother skeletons, although some characteristic diversities are seen between them. Carthamin has one methine group linking two asymmetric polyhydroxy quinoidchalcone moieties at the position of 1 and 1' carbon atoms, and one semi-quinone attached to the carbon-2 of a quinoidchalcone block. Safflor yellow B keeps one glucitol on the saturated bond interposed between two symmetric polyhydroxy quinoidchalcone units at the carbon atoms of 1 and 1' positions (Takahashi et al. 1982, 1984, see Fig. 1a and b).

Thinkable explanations of the restless sensitivities of the quinoidchalcones towards oxidants result from the following reasons: (1) carthamin is oxidatively formed from precarthamin (Saito et al. 1983 a,b, 1985, Saito and Takahashi 1985), which makes it possible to stand against electrophilic attack from oxidants and, in turn, it is unstable towards nucleophilic reagents, (2) safflor yellow B is a product formed

in plant tissues in conditions of O₂ deficiency, which perhaps reflect in the sensitive nature of the pigment to oxidants. The antioxidants used here react also with both carthamin and safflor yellow B differently. Hydroquinone acts on these two pigments as a prooxidant. L-Ascorbic acid shifts carthamin red to a bathochromic side, though it shows no effect on safflor yellow B. The pro-oxidant action of these antioxidants has already been reported in several publications (Watts and Wong 1951, Kanner et al. 1977, Saito and Katsukura 1992c). D-Isoascorbic acid reduces carthamin red colour, while it induces no shift on safflor yellow B (see Figs 9 and 10).

It has frequently been recognized that the complicated and perplexing mechanisms of enzymic reactions can be resolved through studying simple and pure chemical processes. Our recent studies (Saito and Takahashi 1985, Saito 1992a-c, Saito and Katsukura 1992a) shown partially in the above section may provide a suitable example of the documentation. The data have now answered our questions partly why carthamin generating activity spreads widely over the vegetative tissues of dyer's saffron, although, independently of the enzyme distribution, precarthamin and carthamin occur limitedly in the yellowish parts of the tubular flowers (Saito 1992b, Saito and Fukushima 1989c, Saito et al. 1983b, 1989b).

Many results have been well-reported in the literature, indicating that peroxidases are dominant in the catabolism of flavonoids and related compounds (Barz and Hösel 1975, Barz and Wiermann 1981, Barz 1981). However, the indication can not always cover up to whole catabolic mechanisms of the phenolic compounds including quinoidchalcone glycosides under the current topics. Very recently, we have isolated an enzyme from the etiolated seedlings of dyer's saffron which decomposes safflor yellow B (Saito and Katsukura 1992b). The enzyme acts positively on the quinoidchalcone with or without presence of H₂O₂. The optimum pH and SDS electrophoretic pattern are markedly different from those of an authentic horseradish peroxidase sample. The effective metal cofactor seems not be iron (III), but manganese (II), although this important problem is to be re-examined more definitely.

In general, peroxidase activity has routinely been assayed in a colorimetric method by the addition of H₂O₂ and/or iron (III) in the incubation mixture. Still, the present data let us realize to be more prudent in judging the catalytic enzyme mechanism, if only H₂O₂ and/or iron (III) promote the oxidative dissimilation of flavonoids and related compounds. Both oxidants and antioxidants act decisively on the quinoidchalcone transcolour reaction. These findings from the non-enzymatic studies will surely contribute to make us more insight into the catalytic mechanisms of the processes before long.

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WRAŻLIWOŚĆ KARTAMINY I SAFLORU ŻÓŁTEGO B NA SUBSTANCJE UTLENIAJĄCE I REDUKUJĄCE

STRESZCZENIE

Wrażliwość kartaminy i safloru żółtego B na substancje utleniające i redukujące była badana w roztworach zbuforowanych o różnych stężeniach. Testy chemiczne w różny sposób oddziaływały na kolor barwników: (1) zewnętrzne działanie O miało mniejszy wpływ na zmianę koloru kartaminy, (2) pomarańczowożółtej barwy saflor żółty B białiał pod wpływem metanu, (3) kartamina wykazywała małą lub żadną wrażliwość na H_2O_2 przy stężeniu od 0.001-230 mikromoli, (4) saflor żółty B reagował z utleniaczami łatwo tworząc nieznany związek o delikatnie żółtej barwie, (5) dwuznaczne wyniki otrzymano przy traktowaniu roztworami kwasu L-askorbinowego i D-izoaskorbinowego; mianowicie pierwszy z nich intensyfikował barwy kartaminy, drugi zaś powodował zanik barwy czerwonej, (6) hydrochinon wzmacnia czerwone zabarwienie roztworu kartaminy, (7) saflor żółty B ulegał rozdzielowi w chromatografii pod wpływem działania roztworu hydrochinonu, nie działało się tak natomiast w obecności roztworów kwasów L-askorbinowego i D-izoaskorbinowego. Wyniki były analizowane z uwzględnieniem oksydacyjnego rozkładu chinochalkonoidów w tkance kwiatowej w główkach szafranu.

SŁOWA KLUCZOWE: kartamina, saflor żółty B, wrażliwość, substancje redukujące, szafran (*C. tinctorius*)