Apparent expression of flower colours and internal variation of enzyme activities in some typical phenotypes of dyer's saffron cultivars

KOSHI SAITO, AKIYOSHI FUKUSHIMA

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

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Abstract

Phytochemical screening of four *Carthamus* pigments in phenotypically different cultivars of dyer's saffron was carried out by means of chromatographic techniques. The pigment composition in the floral part correlated well with the flower colour, supporting these components as idoneous chemotaxonomic markers. Among seven cultivars examined, three were orange-yellow and contained carthamin (red) and precarthamin, safflor yellow A and safflor yellow B (orange-yellow) (type O). There were bright-yellow and also had the above four pigments (type Y). The seventh cultivar was ivory-white and produced no quinoidal chalcones in the florets (type W). Relative activities of three different enzymes were examined in soluble protein extracts from etiolated seedlings of the garden varieties. Monophenol monooxygenase (EC 1.14.18.1) and peroxidase (EC 1.11.1.7.) were distributed over all cultivars tested. The relative level of the enzyme activities could be ordered as follows: type O, type W and type Y. The activity of a carthamin-synthesizing enzyme was found in the protein extracts from all garden forms examined. Its activity was most prominent in type O. The activity level in type W was inferior to that of type O. The catalytic intensity in type Y was found to even lower. The results were discussed as to the composition of the phenotypic markers and the distribution of the enzyme activities in three different garden forms of dyer's saffron cultivars.

Key words: *Carthamus tinctorius*, dyer's saffron cultivars, pigment composition, enzyme activity

Abbreviations: PC — paper chromatography, TLC — thin-layer chromatography. UV — ultra-violet, IR — infrared, BD — benzoyl diethyl aminoethyl

INTRODUCTION

Indisputable description on the phytochemically and economically contributive cultivars of ornamentals often possess serious problems. Indeed, clear-cut differences between cultivars of a certain species are frequently lacking and this represents usually an important handicap with regard to the unequivocal description and/or recognition of a given cultivar. Fortunately, since recent advances in phytochemical and biochemical techniques, we have recognized the additional potential of plant constituents and certain enzymes for the characterization and classification of plants.

Dyer's saffron, which belongs to a genus of *Compositae* (*Dicotyledoneae*), comprises over seventy varieties on the species (Seegeler 1983, Dr. H. Kanai — personal communication). It can be divided visually into three phenotyps by the floret coloration: the first type is orange-yellow (designated as type O), the second bears bright-yellow florets (designated as type Y) and the third is albino, and the flowers are ivory-white (designated as type W).

Carthamin has been isolated from the mature flowers of an orange-yellow garden form, "Mogami Benibana" in Japanese, which belongs to the type O and its chemical structure is now established spectroscopically (Obara and Onodera 1979, Takahashi et al. 1982). Other orange-yellow saffron pigments have also been exclusively investigated in only limited varieties which are included in the type O. Therefore, it is not surprising that little is known about the pigment composition in other phenotypically distinguishable saffron cultivars. Precarthamin, safflor yellow A and safflor yellow B have been isolated from the Mogami Benibana and characterized partially (Takahashi et al. 1982, 1984a, b, Onodera et al. 1981). Perhaps, quantitative levels of these quinolid chalcones may chiefly contribute to determine the phenotypic characters through the flower colour modification in the cultivars. Each member comprised in the type O is known to change its flower colour from bright-yellow to reddish-orange or dark-red just before its floral wilting stage.

Recently, the flower colour transition has been studied *in vitro* and the mechanism revealed partially, suggesting that precarthamin is converted to carthamin *via* the process of aerobic oxidation reaction (Saito et al. 1983a, b, 1985a, b, Saito and Takahashi 1984, Takahashi et al. 1984b, Homma et al. 1985). Biologically, this reaction is induced by an enzyme or enzyme system(s) tentatively named "carthamin-synthesizing enzyme". Together with carthamin-synthesizing enzyme, the activities of monophenol monooxygenase and peroxidase are easily detected in the protein extracts from the plant material. These are also assigned to polyphenol oxidation and play a great variety of roles in polyphenol metabolism in living systems (Mayer and Harel 1979, Mayer 1987). It seemed to be worth researching on the relationship between the flower colour expression and the enzyme activities in the garden varieties of the ornamentals.

We investigated the distribution of four quinoid chalcones and the catalytic allocation of three different enzymes to examine biosystematic aspects of floral colour variation.

MATERIALS AND METHODS

MATERIALS

Different seed lots of seven phenotypically distinguishable cultivars of dyer's saffron were used. After full blooming, the florets of three cultivars change from orange-yellow to dark-red, they remain to bright-yellow in three cultivars or ivory-white in one. All were grown under a phyletic line culture and harvested late in August, 1984 (designated as seed lot A) and 1987 (designated as seed lot B) and were gifts. Florets were gathered at intervals at full blooming stage of the cultivars from the seed lot B. Seeds from the lot A or B (40 g per tray each) were weighed, rinsed in tap-water for about 15 h and sterilized in 1% sodium hypochlorite for 30 min., and washed with deionized-distilled water. They were sowed on damp vermiculite and kept at 28°C in the dark. The etilated hypocotyl tips were gathered from 3-4-day-old seedlings after sowing and immersed into about 4 cm³ per g fresh weight of 70% aqueous ethanol for 3 min., followed by washing 6-7 times with about 5 cm³ per g fresh weight of deionized-distilled water. Chemicals used for enzyme preparation and its assay were obtained from the same sources as reported previously (Saito et al. 1983a, b, 1985a, b, Takahashi et al. 1984, Homma et al. 1985). Carthamin, precarthamin, safflor yellow A and safflor yellow B used as standard specimens were from our laboratory stocks. Other chemicals and reagents used were of analytical grade obtainable commercially.

EXTRACTION AND IDENTIFICATION OF *CARTHAMUS* PIGMENTS

Florets (30g fresh weight each) from the cultivars were extracted with 3 vol. per fresh weight of 80% aqueous methanol three times at room temperature for each 60-90 min. each time. Combined extracts were filtered and the filtrates evaporated to a small volume. Each concentrate was then dissolved in distilled water (5 ml), acidified with acetic acid to pH 3-3.5, treated with activated BD-cellulose (1 g wet weight) and the arrested matters were recovered by an appropriate amount of acetone/formic acid (8:2, by vol.). The released compounds were condensed with a rotary evaporator at less than 35°C and purified by column chromatography on Avicel cellulose (n-butanol/ethylacetate/methanol/water, 4:4:1:2, by vol.), Saphadex LH-20 (distilled water) and Toyo Pearl HW-40 (methanol/water, 65:35, by vol.). Purified orange-yellow pigments were identified by comparing their colours, R_f -values on PC and

TLC, and the spectroscopic properties with those of authentic specimens through UV and IR. Red carthamin was isolated and identified following the methods reported previously (Saito et al. 1983a, b).

PREPARATION OF ENZYME EXTRACTS

All work was done in a cold room at 2-4°C. Protein extracts were obtained as follows: weight amounts (5.5-38.8 g fresh weight) of the washed seedlings were homogenized in cold 50 mM citrate-phosphate buffer (pH 7.0) containing 20 mM sodium araboascorbate and 0.1 mM 2-mercaptoethanol. The homogenate was squeezed through nylon-cloth and the filtrate centrifuged for 20 min. at $15\,000 \times g$ in refrigeratory centrifuge. The supernatant was fractionated by solid ammonium sulfate and the fraction precipitated between 0 and 80% ammonium sulfate saturation was retained, then treated with Sephadex G-25. The salt free extracts were used for assaying enzyme activities.

ASSAY OF ENZYME ACTIVITIES

For the assay of the carthamin-synthesizing enzyme, the reaction mixtures contained, in a total volume of 1.5 cm³, 91 µg precarthamin, 1 cm³ of protein extract (691-908 µg protein per cm³), 100 mg cellulose powder and 50 mM acetate buffer (pH 4.8). The mixtures were incubated in a water-bath incubator at 30°C for 2 h with gentle shaking (92 strokes per min). At the end of the incubation, the reaction was terminated by the addition of 0.1 cm³ of glacial acetic acid. After washing with 5 cm³ distilled water, the red product was liberated from the damp cellulose with 5 cm³ of 60% aqueous acetone. The content of carthamin formed was estimated from the data obtained by measuring the absorption peak at 521 nm (Saito et al. 1985b).

The activity of monophenol monooxygenase was measured spectrophotometrically by following changes in absorbance at 475 nm for 5 min. at 30°C (Katan and Galun 1975). The reaction mixtures contained 4 mM L-3, 4-dihydroxyphenylalanine, 69.1-90.8 µg protein extracts and 100 mM phosphate buffer (pH 6.7), in a total volume of 2.6 cm³.

Peroxidase activity was estimated at 420 nm using guaiacol as a hydrogen donor and hydrogen peroxide as a hydrogen acceptor, essentially after the method of Tomiyama and Stahmann (1964). The incubation medium was composed of the following components in a final volume of 4.1 cm³: 11 mM guaiacol, 16.5 mM hydrogen peroxide, 69.1-90.8 µg protein and 55 mM phosphate buffer (pH 6.0). The enzyme reaction was carried out at 30°C for 5 min.

Protein contents were determined following the Folin-Ciocalteu method modified by Lowry et al. (1951).

RESULTS

SURVEY OF *CARTHAMUS* PIGMENTS IN AQUEOUS EXTRACTS OF DYER'S SAFFRON CULTIVARS

The data from the qualitative analyses of various saffron pigments in the floral extracts of three different phenotypes (type O, type Y and type W) are summarized in Table 1. Carthamin, precarthamin, safflor yellow A and safflor yellow B are contained in all members of type O. These pigments are also distributed over the floral parts of type Y, although a considerably lower level of the carthamin and precarthamin contents was roughly estimated by marked spots on the dried chromatograms of both PC and TLC. In type W no quinochalcones were detected in the extracts. The difference in the pigment composition correlates well with the phenotypic expression of the flower colour of the garden varieties.

Table 1

Composition of quinoid chalcone pigments in dyer's saffron cultivars

Type	Cultivar	Quinoid chalcone glycoside*			
		CTM	PTM	SFY-A	SFY-B
O	Zairai Wase	+	+	+	+
	Zairai Okute	+	+	+	+
	Mogami Benibana	+	+	+	+
Y	URO	+	+	+	+
	Isuraeru	+	+	+	+
	Saradayu Benibana	+	+	+	+
W	Hakushoku-shu	—	—	—	—

* CTM — carthamin, PTM — precarthamin, SFY-A — safflor yellow A, SFY-B — safflor yellow B.

TEST FOR GERMINATION OF *CARTHAMUS* SEEDS FROM DIFFERENT CULTIVARS

Table 2 presents the germination rate of different seed lots from seven phenotypes of dyer's saffron. 100-200 Seeds selected randomly from seed lots A and B were germinated on moist vermiculite and, after given times of incubation, the dark-grown seedlings with 0.2-6.8 cm long sprouts were counted. The rate averaged from each lot of A or B was 66.3 and 63.9%, which corresponds to following ratio, 1.04:1. The data prove that these seed lots can be used safely for the enzyme preparation.

Table 2
Germination rate of dyer's saffron seeds

Type	Cultivar	Seed lot	
		A*	B**
O	Zairai Wase	88.0	85.0
	Zairai Okute	80.0	74.0
	Mogami Benibana	73.0	29.3
Y	URO	61.0	27.0
	Isuraeru	36.0	82.5
	Saradayu Benibana	61.0	70.0
W	Hakushoku-shu	65.0	79.3

* Harvested in 1984, ** Harvested in 1987.

VARIATION OF ENZYME ACTIVITIES IN THE ETIOLATED SEEDLINGS OF DYER'S SAFFRON CULTIVARS

Carthamin-synthesizing enzyme activity. The activity of carthamin-synthesizing enzyme was measured in the cell-free extracts from the excised hypocotyls of different phenotypes of the dark-grown seedlings (Table 3). The enzyme activity was distributed over all phenotypes examined, regardless of the apparent expression of the flower colour; carthamin or precarthamin productive capacities obviously varied among the test cultivars. The relative activity of the enzyme was higher in the extracts from lot A than in those from

Table 3
Comparison of the catalytic activity of
carthamin-synthesizing enzyme in seedling
extracts from various cultivars

Type	Cultivar	Specific activity (pM carthamin μg protein ⁻¹ min ⁻¹)	
		A*	B**
O	Zairai Wase	81.4	28.0
	Zairai Okute	80.8	31.7
	Mogami Benibana	91.3	104.7
Y	URO	27.8	24.0
	Isuraeru	67.8	60.4
	Saradayu Benibana	77.0	31.7
W	Hakushoku-shu	65.5	25.4

*, ** See Table 2.

lot B. Each averaged value in A or B was as follows: 3.9 and 2.9, which corresponded to the ratio, 1.3:1.0. The catalytic level of carthamin-synthesizing enzyme was the highest in carthamin-producing and orange-yellow cultivars (type O). The ratio in lot A and B was calculated to be 5.8:3.3. The level in type W (albino) followed this (4.2 and 3.3). The carthamin-synthesizing from type Y was lower (1.7 and 2.1).

Monophenol monooxygenase activity. The maximum activity of monophenol monooxygenase in the soluble protein preparations from the test forms is shown in Table 4. Monophenol monooxygenase of seed lot B was usually lower than that of lot A. About two-fold higher level of the catalytic activity was found in the extracts from A. Monophenol monooxygenase in type O kept comparatively high level (0.56), though it varied between cultivars and seed lots to a considerable extent. The specific level of each lot was averaged to be 0.70 (A) and 0.42 (B), respectively. The activity of the enzyme in type Y varied almost randomly both in the varieties and in the seedling batches used. The mean values among three cultivars were 0.48 and 0.16 (see Table 4, column A and B). The catalytic intensity in type W stood well between type O and Type Y. The enzyme activity in these types was also stronger in the lot A than in B.

Table 4
Comparison of the catalytic activity of monophenol monooxygenase in seedling extracts from various cultivars

Type	Cultivar	Specific activity ($\Delta A_{475 \text{ nm}} \mu\text{g protein}^{-1} \text{ min} \times 10^{-3}$)	
		A*	B**
O	Zairai Wase	0.81	0.34
	Zairai Okute	0.77	0.38
	Mogami Benibana	0.52	0.54
Y	URO	0.49	0.23
	Isuraeru	0.23	0.05
	Saradayu Benibana	0.73	0.21
W	Hakushoku-shu	0.67	0.30

*, ** See Table 2.

Peroxidase activity. Table 5 provides data on peroxidase activity distribution in the protein extracts from the various saffron varieties. A marked high specific activity was found in the lot A of type O (34.2 average), although the activity in the same lot varied between type Y (31.9) and type W (26.5). In the lot B, relatively high peroxidase activity localized exclusively in type

O (20.7). Lower level of the enzyme was observed in ivory-white cultivar (albino type). The catalytic strength of peroxidase in bright-yellow varieties tended to decrease more conspicuously (only about 30% of the carthamin-producing cultivars).

Table 5

Comparison of the catalytic activity of peroxidase in seedling extracts from various cultivars

Type	Cultivar	Specific activity ($\Delta A_{420 \text{ nm}} \mu\text{g protein}^{-1} \text{ min} \times 10^{-3}$)	
		A*	B**
O	Zairai Wase	20.7	11.1
	Zairai Okute	48.3	21.1
	Mogami Benibana	33.5	30.0
Y	URO	21.3	9.4
	Isuraeru	37.9	1.2
	Saradayu Benibana	36.4	8.4
W	Hakushoku-shu	26.5	11.8

*, ** See Table 2.

DISCUSSION

Dyer's saffron is unique among *Compositae*, because it produces novel quinochalconoid plant pigments such as carthamin, precarthamin, safflor yellow A., safflorin A and safflor B in the matured florets (Obara and Onodera 1979, Onodera et al. 1981, Takahashi et al. 1982, 1984a, b). Because of their economical significance, the pigment chemistry seems to have been done exclusively in only carthamin-producing and orange-yellow varieties.

In this work distribution pattern of the novel pigments and catalytic variation of three different phenol-oxidizing enzymes were investigated in three typical phenotypes of the herbal plant. Saffron cultivars which belong to type O are deeper in the floret colour than those of type Y. Ancommon cultivar bearing ivory-white florets, which we encounter rarely, contain no quinoid chalcone glycosides as described above. Most of these pigments can be identified by simultaneous PC, TLC and/or direct comparison. Thus the major constituents, carthamin, precarthamin, safflor yellow A and safflor yellow B, have readily been identified in the crude extracts. For more detailed identification it was preferable to determine UV- or IR-spectra. The proportion in which these compounds occur in different cultivars are rather variable, however, it is evident that at least the major components are present and constitute a typical pattern. Sometimes, plants which belong to the same genus can be divided into plural forms through not only its apparent coloration, but

its internal chemosystems. This is supported partially by the fact that the pigment composition correlates well with the floral colour expression of the ornamentals. Quinoid chalcone glycosides are useful as idoneous chemotaxonomic markers in *C. tinctorius* as well as in closely related species such as *C. lanatus*, *C. dentatus*, *C. glaucus* and so on.

The results from the studies on the catalytic variation of phenol-oxidizing enzymes provide us with some complicated problems concerning the characterization and classification of the plants with which closely associated relations. Monophenol monooxygenase and peroxidase are known to be distributed widely over a vast variety of biological systems, and have been assigned many roles in polyphenol metabolism including terminal electron transport, lipid oxidation, amino acid oxidation, glycoside oxidation and various hydroxylations (Mayer and Harel 1979, Mayer 1987). Therefore it is natural that they are detected commonly in living tissue extracts of dyer's saffron cultivars. Apart from two polyphenol-oxidizing enzymes, the case of carthamin-synthesizing enzyme seems to be somewhat different, because it mediates the formation of red carthamin from an orange-yellow precarthamin under aerobic conditions (Saito et al. 1983a, b). The enzyme has been studied *in vitro* systems and the characteristic properties are now partially known (Saito et al. 1985a, b, Homma et al. 1985, Saito et al. 1986, 1988). It is active on acidic site and requires atmospheric oxygen for the optimal reaction. It shows no activity with L-3,4-dihydroxyphenylalanine or with guaiacol and hydrogen peroxide. It contains neither copper nor iron. The activity of the enzyme is inhibited in the presence of ortho-phosphate and enhanced by bivalent manganese. It spreads over the vegetative tissues with or without capacity of carthamin formation and is preferentially associated with the cytosol fraction. Accordingly, the flower colour transition in dyer's saffron belonging to the type O (and perhaps also to the type Y) may be induced if precarthamin is supplied to the catalytic site of carthamin-synthesizing enzyme in the living cells. However, it is still obscure from non-carthamin producible cultivar, type W, though the catalytic intensity varies according to the plant materials harvested from the seed lot supplied (Table 3). Is precarthamin the sole substrate for carthamin-synthesizing enzyme? Doesn't the carthamin-synthesizing enzyme catalyze similar divergent reactions? Is the catabolic activity of carthamin equal among three types? Could there be little or no trace amounts of carthamin synthesized also in type W? Although no precarthamin has been found in the hypocotyl extracts from 5.4 kg fresh weight of Mogami Benibana (unpublished results), these aspects require further detailed studies.

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Zależność między barwą kwiatów i aktywnością enzymów u fenotypowo różnych odmian szafrań

Streszczenie

Metodami chromatograficznymi zbadano cztery barwniki występujące u rodzaju *Carthamus*, u fenotypowo różnych odmian szafrań. Skład barwników kwiatów ściśle zależał od koloru kwiatów, co pozwoliło uznać te barwniki za wskaźniki chemotaksonomiczne. Spośród siedmiu

badanych odmian, trzy były pomarańczowożółte i zawierały kartaminę (czerwona) i prekartaminę oraz safflor żółty A i safflor żółty B (pomarańczowożółty) (typ O). Dalsze trzy odmiany były jasnożółte i również zawierały wymienione cztery barwniki (typ Y). Siódma odmiana była kremowobiała i nie wytwarzała badanych barwników w kwiatach (typ W).

W ekstraktach białek rozpuszczalnych z etiolowanych siewek odmian ogrodowych szafranu badano względną aktywność trzech enzymów. Monooksygenaza monofenolowa (EC 1.14.18.1) i peroksydaza (EC 1.11.1.7) występują u wszystkich badanych odmian. Względny poziom aktywności enzymatycznej można uszeregować następująco: typ O, typ W i typ Y. W ekstraktach białkowych wszystkich badanych odmian stwierdzono aktywność enzymu syntetyzującego kartaminę. Jego aktywność była największa u typu O, mniejsza u typu W i najmniejsza u typu Y.

W dyskusji omówiono skład barwników jako wskaźników fenotypowych oraz rozkład aktywności enzymatycznej u trzech różnych typów ogrodowych odmian szafranu.