Effect of external conditions on the stability of enzymically synthesized carthamin

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Abstract

Enzymically synthesized carthamin was subjected to testing of its stability under various conditions. It easily turned to unknown reddish-yellow or orange-yellow substances in water. This instability was further accelerated by the presence of a sulfhydryl reagent and/or at higher temperature. In a typical result, the rate of the discolouration was calculated to be $2.1 \text{ nmol carthamin h}^{-1}$ at 20°C in water and rose to $44.0 \text{ nmol carthamin h}^{-1}$ at 60°C in water containing 1.0 mM 2-mercaptoethanol. Organic solvents such as methanol, ethanol and acetone showed little effect on carthamin discolouration, while the stability of carthamin in these solvents was gradually decreased by rising temperature. In various buffers, in the acidic range, carthamin was relatively stable, whereas, it turned unstable on the basic side. All of the thiol reagents tested were unfavourable for retaining stability of carthamin in solutions. 2-Mercaptoethanol, reduced glutathione and thioglycolic acid exhibited the strongest degrading effect on the pigment. At the 1.0 mM level, 2-mercaptoethanol reduced the absorption peak at 517 nm by 0.02 min^{-1} , which corresponded to $0.12 \text{ nmol carthamin cm}^{-3} \text{ min}^{-1}$. L-Methionine, L-ascorbic and D-araboascorbic acids were found to be strong bathochromic effectors. For example, L-methionine raised $\Delta A_{517 \text{ nm}}$ up to 0.01 min^{-1} , whose value equaled to $0.1 \text{ nmol carthamin cm}^{-3} \text{ min}^{-1}$ at 30°C . Carthamin was absorbed and adsorbed and stabilized by many macromolecular substances. Among the compounds tested, cellulose was found to be the most effective stabilizer. The stabilization effect of cellulose was specific for carthamin and the term "SAITO EFFECT" is proposed for it. This effect was reduced by hydrolysis of the carthamin-adsorbed cellulose with *Aspergillus* cellulase in an incubation system.

Key words: carthamin, thiol reagent, reducing agent, cellulose, stability effect

INTRODUCTION

Carthamin is unique among flavonoid compounds because it has a characteristic quinochalcone structure in its mother skeleton (Takahashi et al. 1982, Obara and Onodera 1979). The most significant contribution of this phenolytic glycoside may exert a pronounced influence on the colouration of safflower capitula, particularly in the case of the matured types. The intensity of the chromogenic property, in general, is in direct correlation with its concentration in the floral tissues. The maximum effect is revealed in the dark red colour produced by enzymic (Saito et al. 1983a, b) and/or non-enzymic (Saito and Takahashi 1985) oxidation of precarthamin, a direct precursor of the synthetic reaction (Takahashi et al. 1984). Studies on carthamin formation have been done in buffered solutions containing precarthamin and proteinous extracts (Saito et al. 1985a, b, c, Homma et al. 1985) or oxidizing agents (Saito and Takahashi 1985). The rate of the catalytic activities has usually been determined spectrophotometrically by monitoring the initial change in absorbance at 517 nm and then the resulting product is often identified with an authentic carthamin after purifying it by the routinely applied cellulose powder adsorption method (Saito et al. 1983a). The catalytically synthesized carthamin, however, is unstable especially when it is left in aqueous solutions in a free state and readily turns to unknown orange-yellow or yellow substances whose structures have presumably not yet been elucidated no mention in literature has been found. The lability of this compound is further pronounced by contact with 2-mercaptoethanol which has often been utilized in reaction media to protect cysteinyl residues in the enzyme protein from oxidative denaturation. This is practically disadvantageous for spectroscopic determination of the catalytic activity of carthamin formation, for the instability of the synthesized carthamin could make experimental data obscure.

Based on some preliminary observations, we performed the following experiments for testing the stability of enzymatically synthesized carthamin under various conditions. Herein evidence from the current study is presented, suggesting that the stability of the pigment was largely affected by temperature, pH value, solvent system and so on. The data are discussed with regard to a possible mechanism through which carthamin is stabilized.

MATERIALS

Carthamin used exclusively in this experiment was prepared enzymatically and purified by chromatographic techniques as described in a previous communication (Saito et al. 1983a). The chemicals used were: thioglycolic

acid, glutathione (reduced form), 2-mercaptoethanol, 2-mercaptobenzothiazole, 5,5'-dithio-bis(2-nitrobenzoic acid), L-cysteine, and L-ascorbic acid — Wako Pure Chemicals Co., Ltd. (Osaka, Japan); D-arabo-ascorbic acid — Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan); glutathione (oxidized form) — Sigma Chemical Comp. (St. Louis, Mo., USA); N-phenylthiourea and dithiothreitol — Nakarai Kagaku Yakuhin Co., Ltd. (Kyoto, Japan); L-methionine — Ajinomoto Co., Ltd. (Tokyo, Japan); cellulose powder — Macherey Nagel (Doren, West Germany); DEAE- and CM-cellulose — Whatman Ltd. (Kent, England); cellobiose, starch and active carbon — Wako Pure Chemicals Co., Ltd.; Sephadex G-25, LH-20 and DEAE Sephadex A-50 — Pharmacia Fine Chemicals (Uppsala, Sweden); PVP (insoluble polyvinylpyrrolidone) — Sigma Chemical Comp.; Toyo Pearl HW-40F (polyvinyl type adsorbent) — Toyo Soda Co., Ltd. (Tokyo, Japan); hydroxyapatite — BDH Chemicals Ltd. (Poole, England); cellulose of 1,4-(1,3; 1,4)- β -D-glucan 4-glucanohydrolase (EC 3.2.1.4) from *Aspergillus niger* — Sigma Chemical Comp. All other chemicals and reagents used were of the highest purity obtainable. Macro-molecular substances obtained from the above listed commercial sources were washed successively with 0.2 M NaOH, 0.2 M HCl, distilled water and finally ethanol. After these treatments they were dried at room temperature and the resulting dried matters were stocked separately in 10 cm³ vials before use.

METHODS

THE EFFECT OF SOLVENT SYSTEMS ON THE STABILITY OF ENZYMICALLY SYNTHESIZED CARTHAMIN

(A) Test for the stability of carthamin in polar and non-polar solvents

Carthamin (0.01 mg·cm³) in distilled water, methanol, ethanol and 60% aqueous acetone was used separately for spectrophotometric determination.

(B) Test for the stability of carthamin in a buffer containing thiol or reducing agents

Carthamin (5.0 mg) was dissolved in 50.0 mM acetate buffer, pH 4.8 (50.0 cm³). An aliquot of the solution (2.0 cm³) was mixed with 2.0 cm³ 1.0 mM thiol and reducing agents for testing, which were subjected to spectroscopic estimation.

(C) Test for the stability of carthamin in buffers with various pH values

Carthamin (0.11 mM) in 2.0 cm³ of 60% aqueous acetone was mixed with 2.0 cm³ of 50.0 mM buffers with various pH values and used for spectrophotometric assay.

Carthamin stability in solutions from (A) to (C) was determined with a Shimadzu spectrophotometer (type UV 150-02) by measuring the initial change in absorbance at 517 nm for 10 min at room temperature.

THE EFFECT OF TEMPERATURE ON THE STABILITY OF CARTHAMIN IN SOLUTIONS

(A) Test in water

Carthamin (1.0 mg) was dissolved in 25.0 cm³ of distilled water (44.0 μ M carthamin) and the solution was immediately used for spectrophotometric estimation.

(B) Test in water containing 2-mercaptoethanol

To 2.0 cm³ of 22.0 μ M carthamin in distilled water, 2.0 cm³ of 2-mercaptoethanol (1.0 mM in distilled water) were added and the mixture was subjected to spectrophotometric assay.

(C) Test in aqueous acetone

2.0 cm³ of carthamin solution (22.0 μ M carthamin in 60% aqueous acetone) were mixed with 60% aqueous acetone and used for examining colour decrease in the solution.

(D) Test in aqueous acetone containing 2-mercaptoethanol

60% aqueous acetone (2.0 cm³) containing 22.0 μ M carthamin was added to 2.0 cm³ of 60% aqueous acetone containing 1.0 μ M 2-mercaptoethanol. The solution was then used for measuring carthamin decolouration activity.

The stability of carthamin in solutions from above (A) to (D) was separately checked by recording the absorption change at 517 nm after incubation for 30 min at 20, 40 and 60°C. The measurement of decrease in carthamin was performed with a Shimadzu UV 150-02 spectrophotometer and degradative activity of the pigment was determined from the amount of carthamin remaining in the reaction media.

THE EFFECT OF MACRO-MOLECULAR SUBSTANCES ON THE ADSORPTION AND THE STABILITY OF CARTHAMIN

Carthamin (3.3 mg) was dissolved in 100.0 cm³ of 50.0 mM acetate buffer, pH 4.8 (36.3 μ M carthamin). An aliquot (1.5 cm³) of the solution

was immediately mixed with 100 mg of various macro-molecular substances in numbered test tubes. The mixtures were then gently shaken in a water-bath incubator (Yamato, type BT-46) for 24 h at 30°C and the colour change of the substances was monitored with a Shimadzu spectrophotometer after eluting carthamin with 60% aqueous acetone. The amount of carthamin was then calculated from a standard curve.

THE EFFECT OF THIOL REAGENTS ON THE STABILITY OF CELLULOSE-STABILIZED CARTHAMIN

Cellulose powder (100 mg) was suspended in 10 cm³ of 50.0 mM acetate buffer, pH 4.8, containing 9.8 µM carthamin. Then, preincubation was done for 30 min at 30°C in an incubator (Tokyo Rikadenki, type Vapour-mix) with vigorous shaking. The resulting reddish powder was mixed with 2.0 cm³ of 1.0 mM 2-mercaptoethanol, reduced glutathione or thioglycolic acid and incubation continued further for 24 h at 30°C in the incubator. The colour change of the powder was observed after elution of residual carthamin with 60% aqueous acetone.

THE EFFECT OF ENZYMIC HYDROLYSIS ON THE STABILITY OF CELLULOSE-STABILIZED CARTHAMIN

Carthamin-absorbed cellulose powder (100 mg; 98 nM carthamin mg cellulose powder⁻¹) was incubated for 24 h at 30°C with *Aspergillus* cellulase (0.12 units solid⁻¹) in 2.0 cm³ of 50.0 mM acetate buffer, pH 5.0, in a Tokyo Rikadenki mixer with continuous shaking. After completion of the enzyme reaction, the decrease in red colouration was measured at 521 nm in aqueous acetone eluate. The carthamin concentration was computed from a standard curve.

RESULTS

Irreversible colour change of carthamin red in solutions can easily be detected under various external conditions. One of the most effective methods for examining the colour shift is to measure spectrophotometrically the absorption change in solutions. Table 1 (A) shows the temperature-dependence of carthamin colour decrease in aqueous solutions with or without 2-mercaptoethanol. Carthamin was unstable in water at higher temperatures, the instability of which was further enhanced by the presence of 2-mercaptoethanol. In a typical example, the rate of the discolouration was calculated to be 2.1 nmol carthamin h⁻¹ at 20°C in water and it rose to 42.9 nmol carthamin h⁻¹ at 60°C. The higher level of the colour decrease was shown in water with 1.0 mM 2-mercaptoethanol at 20°C

Table 1 (A)

Temperature dependence of colour decrease of carthamin in distilled water with or without 2-mercaptoethanol

Temperature, °C	Incubation time, min	Rate of discolouration, % of control	
		distilled water	2-mercaptoethanol in distilled water
20	10	1.6	56.7
	20	2.2	57.4
	30	2.4	57.9
40	10	7.2	64.6
	20	13.1	71.0
	30	17.6	75.7
60	10	32.5	93.1
	20	44.9	100.0
	30	48.8	100.0

Table 1 (B)

Temperature dependence of colour decrease of carthamin in aqueous acetone with or without 2-mercaptoethanol

Temperature, °C	Incubation time, min	Rate of discolouration, % of control	
		60% aqueous acetone	2-mercaptoethanol in 60% aqueous acetone
20	10	11.8	14.2
	20	25.2	20.5
	30	40.2	65.4
40	10	22.0	24.4
	20	33.1	33.9
	30	35.4	40.9
60	10	43.3	44.1
	20	46.5	93.7
	30	40.2	100.0

(2.1 nmol carthamin h^{-1}) and it shifted to a higher value due to rising temperature. The relative level of decolouration of carthamin in polar and non-polar solvents was shown in respect to the stability of the pigment in the solutions (Fig. 1). Fig. 1 shows that carthamin was unstable in distilled water, while it was relatively stable in the non-polar solvents tested. The stability of carthamin in non-polar solvents, however, decreased progressively with rising temperature. An example is shown in Table 1 (B). These data prove that the rate of colour decrease in

Table 2

Effect of exogenous substances on the stability of carthamin in a buffer solution

Compound	Carthamin remaining, $\text{nmol} \cdot \text{cm}^{-3} \cdot 10 \text{ min}^{-1}$	Rate of colouration, % of control	Rate of discolouration, % of control
2-Mercaptoethanol	3.5	—	93.7
Thioglycollic acid	38.4	—	30.0
Glutathione (reduced form)	42.2	—	23.3
Glutathione (oxidized form)	46.7	—	14.9
L-Cysteine	45.6	—	17.5
Dithiothreitol	54.2	—	1.3
2-Mercaptobenzothiazole	53.9	—	1.9
5,5'-Dithio-bis(2-nitrobenzoic acid)	47.3	—	13.9
L-Cystine	55.7	1.5	—
N-Phenylthiourea	55.2	0.5	—
L-Methionine	64.1	16.7	—
L-Ascorbic acid	63.1	15.0	—
D-Araboascorbic acid	64.0	16.5	—

Table 3

Effect of buffer systems on the stability of carthamin under various pH values

Buffer system	Rate of discolouration, % of control								
	pH value								
	4.0	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5
Acetate	10.0	12.7	—	16.7	—	—	—	—	—
Citrate	2.5	4.8	—	5.6	—	—	—	—	—
Succinate	12.1	17.0	—	28.2	—	—	—	—	—
Phthalate	3.1	3.2	—	7.5	—	—	—	—	—
Maleate	1.4	3.6	—	16.1	—	—	—	—	—
Phosphate	—	0.9	—	2.2	—	—	—	—	—
Tris-maleate	—	13.7	25.9	36.6	42.9	45.8	53.8	66.7	83.2

carthamin is also promoted by addition of 2-mercaptoethanol to the solvent. The red pigment is very sensitive to many thiol and reducing compounds (Table 2). Among the compounds examined at the 1.0 mM level, 2-mercaptoethanol, thioglycollic acid and reduced glutathione showed the most prominent effect on the decolouration of carthamin. L-Methionine, L-ascorbic and D-araboascorbic acids, on the other hand, exerted their attributive bathochromic effects on the red colouration of the carthamin solutions. The pigment can keep its stability to some extent on the acidic side of buffers, whereas, it turns readily to yellow compounds in basic solutions, which manifested itself in the decrease in absorbance at 517 nm (Table 3).

Table 4

Effect of macro-molecular substances on the adsorption of enzymically synthesized carthamin

Macro-molecular compound	Rate of adsorption, % of control
Cellulose powder	97.4
CM-cellulose	97.4
DEAE-cellulose	99.1
Cellobiose	92.2
Starch	96.9
Sephadex G-25	97.0
Sephadex LH-20	77.2
DEAE Sephadex A-50	98.5
Active carbon	99.2
Hydroxyapatite	91.3
PVP	98.3
Toyo Pearl HW-40F	96.4

Macro-molecular substances for testing (100 mg each) were suspended in distilled water (15 cm³) containing carthamin (49.5 µg) and incubated at 30 °C. After 30 minutes of incubation, the carthamin-adsorbed compounds were extracted with 60% acetone and the resulting clear extracts subjected separately to spectrophotometric determination of the carthamin contents as described in Materials and Methods.

Table 5

Effect of macro-molecular substances on the stability of enzymically synthesized carthamin

Macro-molecular compound	Rate of carthamin remaining, % of control	Stability*
Cellulose powder	93.5	26.2
CM-cellulose	16.4	4.6
DEAE-cellulose	5.3	1.9
Cellobiose	12.5	3.5
Starch	51.2	14.3
Sephadex G-25	35.1	9.8
Sephadex LH-20	13.8	3.9
DEAE Sephadex A-50	3.1	0.9
Active carbon	66.7	18.7
Hydroxyapatite	8.9	2.5
PVP	39.2	11.0
Toyo Pearl HW-40F	11.5	3.2

* Stability exhibits the rate calculated from the sum of carthamin remaining (% of control)

Carthamin is adsorbed by many macro-molecular substances. Data from the studies on the adsorption of carthamin to various compounds are summarized in Table 4. After incubation for 30 min at 30°C, 91–99% of carthamin was adsorbed by the compounds tested, although only Sephadex LH-20 showed less affinity for carthamin under the same

Fig. 1. Stability of carthamin in polar and non-polar solvents. Carthamin (1.0 mg) was dissolved in 100 cm³ each of distilled water, methanol, ethanol or 60% aqueous acetone and the colour change in the solvents was separately recorded with a spectrophotometer as described in Materials and Methods

conditions. Carthamin is stabilized after being adsorbed by some macro-molecular adsorbents. In the present study cellulose proved to be the most prominent stabilizer for carthamin (Table 5). Active carbon, starch, Sephadex G-25 and PVP came next. Cellobiose, CM-cellulose, DEAE-cellulose, Sephadex LH-20, DEAE Sephadex A-50, hydroxyapatite and Toyo Pearl were all less effective for carthamin stabilization. Table 6 summarizes the data from observations on the effect of 2-mercaptoethanol, thioglycolic acid and reduced glutathione on the stability of carthamin adsorbed and stabilized by cellulose. The discolouration effects of these compounds are clearly shown to be reduced by the presence of cellulose as compared with the data of Tables 1 (A) and (B). Hydrolysis with *Aspergillus* cellulase makes the cellulose-stabilized carthamin unstable (Table 7). Under

Table 6

Effect of thiol reagents on the stability of cellulose-stabilized carthamin

Thiol reagent	Rate of carthamin disappearance, % of control
2-Mercaptoethanol	26.5
Thioglycollic acid	58.2
Glutathione (reduced form)	48.0

Table 7

Effect of enzymic hydrolysis on the stability of cellulose-stabilized carthamin

Experiment	Decrease in the amount of carthamin, μM carthamin μg cellulose ⁻¹ unit cellulase ⁻¹ h ⁻¹
1	120.4
2	377.9
3	506.3

Under the conditions of the present study, 88 mg cellulose powder was found to be hydrolyzed during the course of the enzyme reaction

the condition of the present study, the rate of carthamin degradation was roughly estimated to be $334.9 \mu\text{M}$ carthamin h^{-1} unit cellulase⁻¹ μg cellulose⁻¹, the average of three different experiments.

DISCUSSION

Free carthamin is unstable especially when left in polar solvents. In a preliminary observation we found that this pigment was easily decolourized in distilled water and turned to unknown reddish-yellow or orange-yellow substances within a day or two at 20–23°C. The decrease in red colouration was accelerated further in the presence of millimolar concentrations of 2-mercaptoethanol and/or at a higher temperature. These facts often hindered us in the estimation of the carthamin content in reaction media used for assaying a carthamin-synthesizing enzyme activity. These unexpected results led us to perform this investigation to obtain some insight into the mechanism by which carthamin becomes unstable. Data from the present studies indicate that the pigment contains extremely sensitive site(s) against hydrophilic or nucleophilic attack from exogenous substances, though the labile site(s) remains obscure. Temperature promotes the discolouration reaction in solvents, the fact of which has

been revealed by a higher rate of decrease in carthamin red with temperature escalation. The instability of carthamin under varied temperature could conceivably result in part from promotion of nucleophilic attack by exogenously applied substances including anionoid reagents. Among the compounds tested, L-methionine, L-ascorbic and D-araboascorbic acids exhibited the reverse effect on the carthamin discolouration. The mechanism through which their bathochromic actions are exerted could not be clarified in this study. Basic conditions make carthamin labile. In general, flavoids are known to be unstable in alkalies and, therefore, the instability of carthamin on the basic side does not seem to be specific for the pigment.

Carthamin is adsorbed by many macro-molecular substances in aqueous solutions. Among these adsorbents, cellulose is the most effective stabilizer for carthamin and the cellulose-stabilized carthamin resists strong nucleophilic attack from reagents known to be typically anionic such as 2-mercaptoethanol, thioglycolic acid, reduced glutathione and so on. The sensitive site(s) of the pigment might be protected by the formation of a tightly fixed linkage(s) with the cellulose molecule presumably through a hydrogen bond. The affinity of carthamin to cellulose is lowered by hydrolysis with *Aspergillus* cellulase. The data suggest that the stability of carthamin on cellulose is closely related to the molecular size of the adsorbents and/or their forms. This is strongly supported by the fact that active carbon, starch, cellobiose and other polysaccharide derivatives are less effective stabilizers for carthamin. The cellulose stabilization is specific for carthamin and no other synonymous safflower pigments including precarthamin show this characteristic property. We propose here the term "SAITO EFFECT" for this specific interaction to form a stable binding between carthamin and cellulose. The cellulose adsorption method based on their specific interaction can be applied as one of the most useful techniques for purification of carthamin on a semi-industrial scale.

Carthamin has been used by the local populations of some countries as a useful dye for cotton cloth for a long period of time. Beautiful red coloured cottons have often been found in articles left for the deceased in ancient tombs and most of the red colour has been proved to be composed of carthamin red. Even for over 2000 years, the carthamin red has remained almost unchanged. The intermolecular binding between carthamin and cellulose is so stable and the cellulose stabilization effect on carthamin is so fascinating!

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Wpływ warunków zewnętrznych na stabilność syntezowanej enzymatycznie kartaminy

Streszczenie

Badano stabilność syntezowanej enzymatycznie kartaminy w różnych warunkach. W wodzie przekształcała się ona łatwo w nieznane czerwonożółte lub pomarańczowożółte substancje. Ta niestabilność była przyspieszana przez odczynnik sulfhydrylowy i lub przy wyższej temperaturze. W typowym przypadku, wielkość odbarwienia wynosiła 2,1 nmoli kartaminy na godz. w 20°C w wodzie i wzrastała do 44,0 nmoli kartaminy na godz. w 60°C w wodzie z 1,0 mM 2-merkaptetanolu. Rozpuszczalniki organiczne, takie jak metanol, etanol i aceton miały mały wpływ na odbarwianie kartaminy, podczas gdy stabilność kartaminy w tych rozpuszczalnikach stopniowo zmniejszała się wraz z wzrostem temperatury. W różnych buforach, o pH kwasowym, kartamina była stosunkowo stabilna, natomiast stawała się niestabilna w buforach zasadowych. Nie udało się utrzymać stabilności kartaminy w roztworach przez użycie różnych odczynników tiolowych. 2-Merkaptetanol, zredukowany glutation i kwas tioglikolowy najsilniej odbarwiał kartaminę. 2-Merkaptetanol, w stężeniu 1,0 mM, redukował szczyt absorpcji przy 517 nm o 0,02 na minutę, co było równoważne 0,12 nmoli kartaminy na cm³ na min. L-metionina, kwas L-askorbinowy i kwas D-arabo-askorbinowy okazały się silnymi efektorami batochromicznymi. Na przykład, L-metionina spowodowała wzrost ΔA 517 nm do 0,01 na min., co było równoważne 0,1 nmoli kartaminy na cm³ na min. w 30°C. Kartamina była adsorbowana i stabilizowana przez

wiele substancji makromolekularnych. Spośród badanych czynników, celuloza okazała się najbardziej efektywnym stabilizatorem. Efekt stabilizujący celulozy był specyficzny dla kartaminy i zaproponowano nazwać go **"efektem Saito"** ("Saito effect"). Efekt ten zmniejszał się podczas hydrolizy celulozy z zaadsorbowaną kartaminą przez celulazę z *Aspergillus* w systemie inkubacyjnym.