Acta Bot. Croat. 57, 123–136, 1998

CODEN: ABCRA 25
ISSN 0365–0588

UDC 577-12

THE TINCTORIAL STABILITY OF CARTHAMIN ON POLYSACCHARIDES AND RELATED SUBSTANCES

KOSHI SAITO

Saito Phyto–Science Institute, Biochemical Laboratory, Phyto-Dye Division, Sapporo, Japan

Various saccharides and related substances were mixed with carthamin in aqueous solutions and their effects on red colour stability compared. At a low temperature range (5 °C), mono- and disaccharides contributed to preservation of the carthamin red colouration. No mono- and disaccharides could relieve carthamin from being bleached at a high temperature (30 °C). Among polysaccharides and their derivates (tested at 30 °C for 24 h in the dark), Avicel cellulose was most effective for the retention of the red colour of carthamin (52 %). Chitin was also promising (41 %). CM-Cellulose, Sephadex G–15 and starch followed this (25, 17 and 13 %, respectively). The effects of alginic acid, chitosan, hyaluronic acid, agar and xylan were still further reduced (11, 8, 8, 7, and 5 % respectively). The adsorption of carthamin towards filter papers was investigated as a function of temperature, pH, buffer concentration and buffer types. To adsorb the red dye effectively, low temperature and high pH were found to be preferable. Buffer types were also contributive. Carthamin adsorption in citrate buffer at pH 4.5 with four different molar concentrations was not significant (24.7 –20.9 %), if compared with data without any buffer added (20.8 %). FT–IR spectrometric analyses of carthamin adsorbed cellulose indicated that hydrogen bonding was involved in the carthamin/cellulose interaction. Based on the data, a hypothetical scheme to characterize a stable binding formation between carthamin and cellulose called the SAITO EFFECT was proposed.

Key words: carthamin, dye, polysaccharide, cellulose, monosaccharides, stabilizers, colouration, textile, food

Introduction

Carthamin, a *Carthamus* quinoidal chalcone glycoside, is a well-known red dye-stuff which has long been used for the colouration of textile fablics and cosmetic goods. In Japan, the dye has also been increasingly applied to a colouring of processed foods in the last two or three years. It dyes food materials red and keeps its fine tincture through a specific interaction known as the SAITO EFFECT. In particular, when the materials to be dyed contain cellulose and related substances, the royal red colouration is retained for a long time (SAITO and FUKUSHIMA 1986). The dye is soluble in aqueous media, but its colouration is sensitive to various external factors and is bleached readily: temperature, pH, UV-light, dissolved oxygen, metal ions and certain chemicals all influence the stability of carthamin dye (SAITO and FUKUSHIMA 1987, KANEHIRA et al. 1990, SAITO and FUKUSHIMA 1991, SAITO et al. 1993a, b, SAITO and MORI 1994a–c, SAITO et al. 1995). To protect carthamin red colour from serious bleaching, insoluble stabilizers are, in most cases, added to the coloured media (SAITO and FUKUSHIMA 1986, SAITO et al. 1993a, SAITO and MIYAMOTO 1994). Among the stabilizers, cellulose appears to have the greatest affinity for the pigment.

With the view of substantiating this claim, comparative studies have been carried out using a series of synthetic glycosyl polymers and a possible mechanism has been suggested: one of the most reactive sites is the primary alcoholic hydroxyl on the glucose units of the macromolecules (SAITO 1990). Although carthamin has many interesting aspects in tinctorial properties appearing after it is adsorbed to natural or synthetic polymers, no systematic studies disclosing the chemical interaction between carthamin and glycosyl polymers have yet been done.

Taking this into consideration, it seemed to be prudent to conduct more detailed studies on tinctorial nature, stabilization, simplified application and practical utility, through which the scientific and/or economic value of carthamin dye could be explored further. The aim of the present study is to characterize the stable tinctorial property of carthamin through the SAITO EFFECT on glycosyl polymers.

Materials and Methods

Materials

Carthamin used in this study as a standard marker was prepared from dried flowers of a dyer's saffron cultivar (Benibana, *Carthamus tinctorius* L., *Compositae*) according to the method of SAITO et al. (1992). D- or L-form of arabinose, D- or L-form of xylose, D- or L-form of ribose, D- or L-form of galactose, 2-deoxy-D-ribose, D-fructose, D-glucose, maltose, cellobiose, lactose, sucrose, starch, chitin, chitosan, agar, citric acid, sodium citrate, potassium phthalate, sodium phosphate (mono- and dibasic), sodium acetate, acetic acid, HCl and acetone, all of which are of analytical grade, were purchased from Wako Pure Chemical (Osaka, Japan). CM–Cellulose and Whatman No. 1 filter papers were

Abbreviations: UV = ultraviolet light, VIS = visible light, IR = infrared light.

purchased from Whatman (Maidstone, England). Avicel cellulose was purchased from Asahi Kasei Kogyo (Tokyo, Japan). Sephadex G–15 was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Xylan from a silver birch (*Betula platyphylla* Sukatchev. var *japonica* Hara) was the gift of Dr. Hata. Alginic acid from two marine algae (*Lessonia nigrescens* and *Lessonia flavicana*) and hyaluronic acid from a streptococcal bacterium (*Streptococcus zooepidemicus*) were the gift of Dr. Murata. Other chemicals and reagents used were obtained from Kanto Kagaku Kogyo (Tokyo, Japan), Nakarai Kagaku Yakuhin (Kyoko, Japan), Merck (Darmstadt, Germany) and Funakoshi Yakuhin (Tokyo, Japan). Insoluble polysaccharides (30–80 g) were washed 5–7 times successively with 0.2 M NaOH, 0.2 M HCl and then distilled water. They were dried in an air circulation oven at 60 °C overnight and stocked in a desiccator over silica gel just before experimental use.

Instruments

A Hitachi UV/VIS double-beam spectrophotometer, model U–1100 (Hitachi Seisakusho, Tokyo) was used. It was connected with a water-bath, EYELA Uni Cool, type UC-55 (Tokyo Rikakiki, Tokyo) and a thermostat from which temperature-controlled water was circulated continuously. The pH measurements were undertaken using a Horiba digital pH meter, model M-8e (Horiba Seisakusho, Tokyo). Incubation was carried out in a water-bath incubator, TAITEC, Personal t-10 connected with TAITEC Lt-100 thermostat (Taiyo, Tokyo).

Incubation of carthamin with simple sugars

Each 5 μmol of sugar (mono- and disaccharide) solution (5 mL) was mixed with 220 μmol carthamin in 100 mM citrate buffer, pH 3.0 and incubated at 5 or 30 °C for 24 h in the dark. The change in the optical density of the carthamin solution was monitored at 521 nm. VIS spectrophotometric readings were used to determine carthamin concentration according to SAITO and MORI (1994b):

$X = Y/0.05855 - 0.000552$ (X: carthamin concentration, μg/mL; Y: optical density at 521 nm).

Incubation of carthamin with polysaccharides and related substances

Carthamin (220 μmol) in 100 mM citrate buffer, pH 3.0 (5 mL) was stirred for 60 min at 30 °C with 100 mg each of Avicel cellulose, CM-cellulose, Sephadex G-15, starch, chitin, chitosan, xylan, agar, alginic acid and hyaluronic acid. The mixtures were then kept in the dark at 30 °C for 24 h in a water-bath incubator with no agitation.

Recovery of carthamin

Each test polysaccharide and related substance from the above experimental process was suspended in 10 mL distilled water, stirred, then transferred to glass tubes and centrifuged at 4 000 × g for 10 min to remove supernatant. The washing process was repeated twice with 10 mL fresh water each time. The washed

polymers were suspended in 25 mL of 60 % (v/v) acetone, stirred for 5 min on a touch mixer at 22 ± 1 °C and the resulting supernatant has collected by centrifugation (4 000 × g, 10 min).

Estimation of carthamin stability

The stability of carthamin on test compounds was estimated spectrophotometrically following the method of SAITO et al. (1992), where 100 mM citrate buffer, pH 3.0 or 60 % (v/v) acetone were used as reference. The data thus obtained were applied to determine carthamin stability through the following equation:

(A – a)/A × 100 % (A: initial carthamin concentration, a: carthamin concentration after incubation).

The stabilities of carthamin on insoluble polymers were calculated by consulting the adsorption and recovery of carthamin. All data listed in figures and tables were averaged from four to five separate experiments.

Test for the adsorption of carthamin towards cellulose

Before the experiments, Whatman No. 1 filter paper (46 × 57 cm) was cut out to a circle (diameter = 6.0 mm, thickness = 0.17 mm, weight = 3.0 mg). The paper was placed in the bottom of a cuvette, into which a test solution was added carefully. The cuvette containing a peace of paper and the test solution was used directly in the spectrophotometric recording process. The test solution contained, unless otherwise indicated, 264 μmol carthamin, 50 mM citrate buffer, pH 4.5 in a total volume of 3.0 mL.

IR spectral measurement

Micro KBr disks and a Degilab, model FT-15E spectrophotometer (resolving power = 8 cm^{-1}) was used for recording the IR spectra. The scanning frequencies were 100 cm^{-1} and reference was air. KBr disks used were: KBr (200 mg)/carthamin (0.5 mg)/Avicel cellulose (2.1 mg) or KBr (200 mg)/Avicel cellulose (2.7 mg). Prior to the experiments, carthamin was mixed with Avicel cellulose in distilled water and the mixtures were dried carefully until the dry weight was a constant value. KBr disks were then prepared using the dried carthamin/Avicel cellulose.

Results

Influence of monosaccharides on carthamin stability after incubation for 24 h at 5 and 30 °C

Table 1 shows the influence of monosaccharides on the stability of carthamin after incubation for 24 h at 5 and 30 °C. When carthamin was incubated in monosaccharide-containing solutions at 5 °C in the dark, monosaccharides showed protective effects against red colour bleaching. The net red colour retention observed at 521 nm was (D-form, %): arabinose (26.3), galactose (10.9), fructose (9.9), 2-deoxy-D-ribose (9.7), xylose (8.7) and so on. D-Forms seem to be

more efficient than L-forms in their ability to stabilize the pigment colouration (average retention 10.9 and 6.4 %, respectively). On incubating carthamin in monosaccharide solutions for 24 h at 30 °C in the dark, the decrease in pigment concentration was pronounced compared with the average data from experiments performed at 5 °C. Net retention of pigment observed in D- and L-forms was (%): 0.06 and 0.28, respectively. At 30 °C, no red colour could be retained in monosaccharide solutions, providing only low rates and/or negative retention values for carthamin (Tab. 1).

Tab. 1. Effect of monosaccharides on carthamin stability after incubation for 24 h at 5 and 30 °C in the dark

Monosaccharide	Net retention* (%)			
	A		B	
	D-Form	L-Form	D-Form	L-Form
Arabinose	26.3	5.3	–0.3**	0.2
Xylose	8.7	6.0	0.2	0
Ribose	7.1	8.0	0.1	0.6
2-Deoxy-D-ribose	9.7	–***	–0.3	–
Fructose	9.9	–	0.3	–
Galactose	10.9		–0.1	0.3
Glucose	3.8		0.5	–

* R-r (R: average rate (%) of red colour retention observed from four to five measurements. r: average rate (%) of red colour retention observed in blank run)
** retention below blank run
*** not determined
A: incubation at 5 °C, B: incubation at 30 °C

Influence of disaccharides on the carthamin stability after incubation for 24 h at 5 and 30 °C

Carthamin incubated in solutions containing disaccharides at 5 °C for 24 h in the dark led to a considerable level of retention of pigment colouration (Tab. 2). The net retention observed was (%): maltose (25.6), lactose (14.8), sucrose (10.2) and cellobiose (7.3). No disaccharides could protect carthamin from being seriously bleached at 30 °C over 24 h incubation (net retention 0.53 % on average).

Influence of polysaccharides and related substances on carthamin stability

To evaluate carthamin stability, carthamin/polymer mixtures were kept at 30 °C for 24 h in the dark. The highest level of pigment was stabilized on Avicel cellulose (net retention 51.6 %). Chitin came next (40.7 %). CM-Cellulose and Sephadex G-15 followed this (24.5 and 16.6 %, respectively). Starch and alginic acid showed more reduced values (13.3 and 10.5 % respectively). Chitosan,

hyaluronic acid, agar and xylan also retained the red colour, but at far lower levels (7.7, 7.5, 6.8 and 5.2 %, respectively) (Tab. 3).

Tab. 2. Effect of disaccharides on carthamin stability after incubation for 24 h at 5 and 30 °C in the dark

Disaccharide	Net retention (%)	
	A	B
Maltose	25.6	0.1
Cellobiose	7.3	−0.3*
Lactose	14.8	1.5
Sucrose	10.2	0.8

* retention below blank run
A: incubation at 5 °C, B: incubation at 30°C.

Tab. 3. Stability of carthamin on polysaccharides and related substances after incubation at 30 °C for 24 h in the dark

Compound	Net retention (%)
Avicel cellulose	51.6
CM-Cellulose	24.5
Sephadex G-15	16.6
Starch	13.3
Chitin	40.7
Chitosan	7.7
Agar	6.8
Xylan	5.2
Alginic acid	10.5
Hyaluronic acid	7.5

Influence of external factors on the adsorption of carthamin on cellulose

Figure 1 shows the change in adsorption of carthamin to a circular piece of filter paper during incubation at various temperature ranges. The carthamin solution bleaches faster in the presence of paper. The relative rate and amount of pigment adsorbed increase at higher temperatures, e. g. 45 °C.

Figure 2 illustrates the influence of the pH on the adsorption of carthamin to a paper piece during incubation for 60 min at 45 °C. Low pH values are effective in promoting carthamin adsorption only after a certain interval of incubation time. Specific values of carthamin adsorption towards pieces of paper at different pH values were (μmol carthamin/mg paper/min): at 1.5 = 0.144, at 2.5 = 0.117, at 3.5 = 0.106, at 4.5 = 0.113, at 5.5 = 0.146, at 6.5 = 0.076, at 7.5 = 0.084.

Carthamin adsorption was examined in citrate buffer (pH 4.5) with four different molar concentrations. Apparent adsorption rates after 60 min calculated

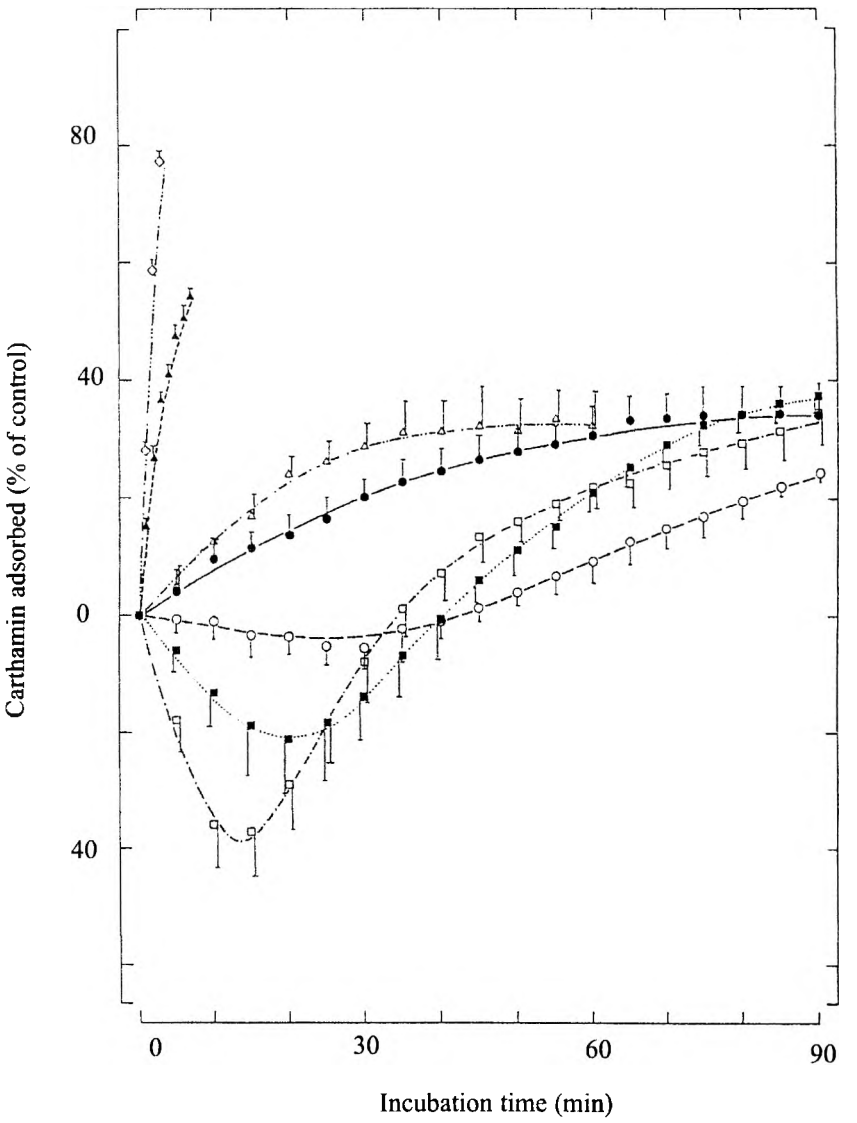

Fig. 1. Effect of temperature on the adsorption of carthamin towards cellulose. A circular filter paper (diameter: 6.0 mm, thickness: 0.17 mm, weight: 3.0 mg) was placed in the bottom of a cuvette. Three millilitres of citrate buffer (50 mM, pH 4.0) containing 264 μmol carthamin were poured carefully into the cuvette and incubated for up to 90 min. Adsorbance was monitored at 521 nm during the course of incubation. The spectrophotometric recording data were used to calculate adsorption rates of carthamin towards cellulose. Blank runs were carried out using 50 mM citrate buffer, pH 4.0 with no addition of carthamin or with addition of carthamin in the absence of paper.

■ ····· ■ : 5 °C, □ -·-·-·-·- □ : 15 °C, O - - - - - O : 25 °C, ● ——— ● : 35 °C,
△ ·· ·· ·· △ : 45 °C, ▲ - - - - - - ▲ : 65 °C, ◇ -···-···-···- ◇ :85 °C.

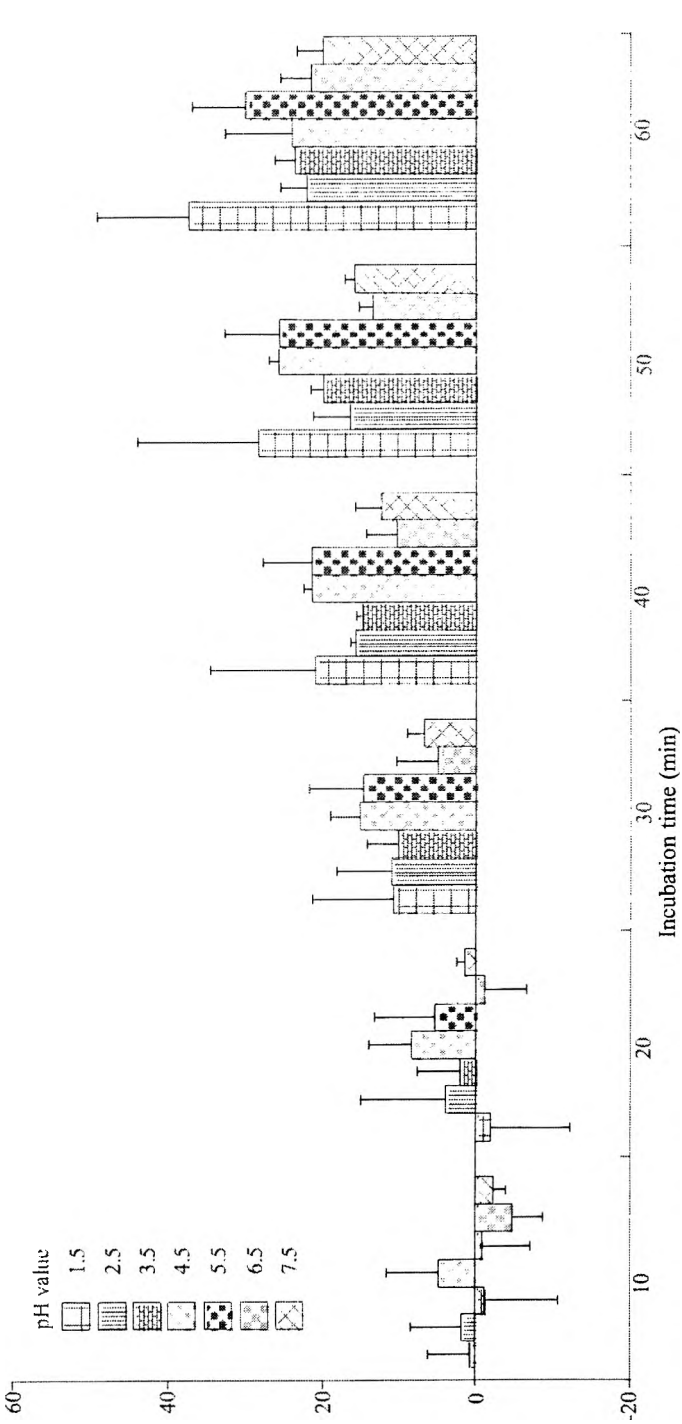

Fig. 2. Effect of pH on the adsorption of carthamin towards cellulose. The following 50 mM buffers were used: KCl/HCl (pH 1.5), phthalate (pH 2.5), citrate (pH 3.5–5.5), phosphate (pH 6.5–7.5). Reactions were carried out at 30 °C for 60 min. Three millilitres containing carthamin (264 μmol each) were poured into the cuvette, in which a circular paper (diameter: 6.0 mm, thickness: 0.17 mm, weight: 3.0 mg) was placed. Absorbance was monitored at 521 nm during the course of incubation. The spectrophotometric recording data were used to calculate adsorption rates of carthamin towards cellulose. Blank runs were carried out using buffers as indicated above with no addition of carthamin or with the addition of carthamin in the absence of paper.

from the experimental data were (% of control): 5 mM (24.7), 100 mM (23.2), 200 mM (22.8), 50 mM (20.9), 0 mM (20.8).

Three buffer types, all at 50 mM and pH 4.5, were evaluated for their effects on carthamin adsorption by cellulose over 60 min at 30 °C. Apparent pigment retention (% of control) was 31.1 for citrate/phosphate buffer, 27.2 for citrate buffer and 22.0 for acetate buffer.

FT–IR spectroscopy of carthamin adsorbed on cellulose

To obtain information on the mechanism for the adsorption and stabilization of carthamin on cellulose, FT-IR spectral analyses were performed. The FT-IR spectra of carthamin adsorbed cellulose are depicted in Fig. 3. Several sharp bands appeared at about 1600 and 1500–1350 cm^{-1}. Spectra for carthamin and cellulose alone show no such characteristic adsorption bands. The data indicate that carthamin interacts with cellulose through chemical bonding.

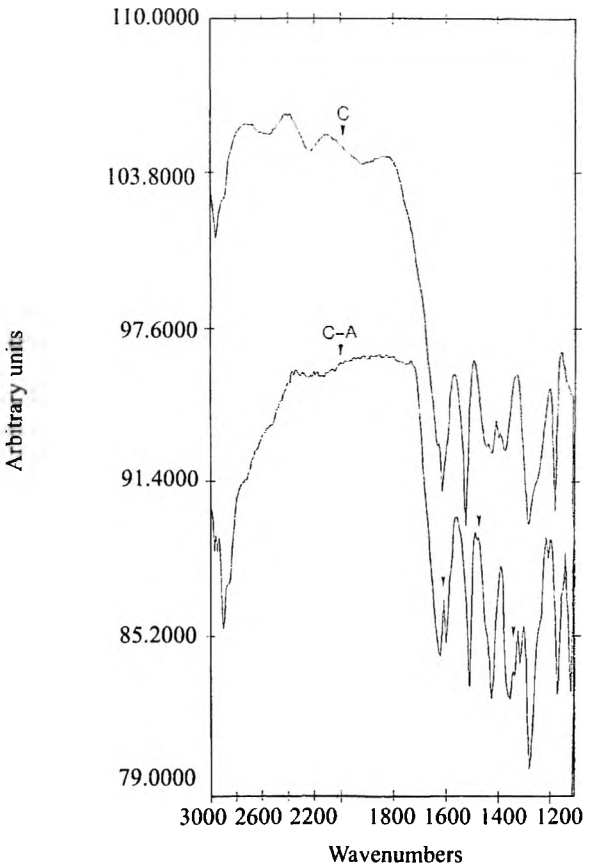

Fig. 3. IR spectra of carthamin with or without being adsorbed on cellulose. C: carthamin, C-A: carthamin adsorbed on cellulose. Carthamin (0.5 mg) was mixed with Avicel cellulose (2.1 mg) in deionized/distilled water and dried to show a constant dry weight. The carthamin/Avicel mixtures were used to prepare micro KBr disks as described in Materials and Methods.

Discussion

The stability of carthamin is affected by various additives and/or external factors. At a low temperature, sugars act on carthamin red colouration protectively. Monosaccharides are slightly more promising stabilizers than disaccharides. Tests of carthamin stability at a high temperature demonstrated that no mono- or disaccharides could reduce the carthamin bleaching rate. To reveal the mechanism of adsorption and /or stabilization of carthamin, further model experiments have been performed at high temperature ranges, where carthamin usually shows labile properties (KANEHIRA et al. 1990, SAITO and FUKUSHIMA 1991). A net weight of carthamin was mixed with the test polysaccharides and related substances in a buffer solution at pH 3.0, and the mixtures were incubated at 30 °C for 24 h in the dark. The data thus obtained clearly show that pigment adsorption was highest in Avicel cellulose. Chitin, CM-cellulose and Sephadex G-15 were also promising. The varied adsorption capacities of the test polysaccharides and related substances are perhaps dependent upon the differences in binding properties, i. e. the facilitated hydrogen bonding ability. Avicel cellulose, chitin and CM-cellulose stabilized carthamin most effectively, as previously shown by SAITO and FUKUSHIMA (1986, 1988), and by SAITO (1990). Under the conditions of the present study, the net retention of carthamin on Avicel cellulose is calculated to be about 51.6 %. Cellulose is constructed by glucose units, which are condensed to form a simple and long β-1,4 glucan straight chain.

Chitin also contributes to carthamin red colour preservation in a promising manner. The polymer retains the fine red colouration after adsorption of carthamin (net retention 40.7 %). Chitin is composed of N-acetylglucosamine chain. It stabilizes carthamin perhaps through the same mechanism as Avicel cellulose. On this occasion, the carbonyl groups of the carthamin molecule must take part directly in the chemical binding process to induce a non-convalent bond with hydrogen on the alcoholic hydroxyls of chitin molecule.

CM-Cellulose is a weak stabilizer for carthamin. It keeps a reddish tincture (net retention 24.5 %). The ion-exchanging cellulose may attract carthamin ionically, in addition to hydrogen bond formation as with the Avicel cellulose, which results in the exhibition of a sizable adsorption and/or stabilization capacity of the pigment administred. Phenolic compounds, including carthamin, are known to change their charges with an ease related to the pH of the polar solvents (HAVLIKOVÁ and MIKOVÁ 1987). In acidic media, they are generally cationic. This probably introduce them to the ionic attraction processes by CM-cellulose. However, the comparatively lower level of carthamin adsorption and/or stabilization on the cellulose cation exchanger could be introduced by the somewhat unfavourable nature of the carboxymethyl radicals.

Distinct and marked differences are seen in the carthamin stabilization capacity on chitosan. The red colouration on chitosan was easily faded and the polymer itself became amber colour after 24 h, although a faint reddish carthamin solution could be recovered by acetone extraction (net retention 7.7 %). Chitosan is a deacetylation product of chitin. Hence its acetyl group at C-2 of glucopyranosyl linkage may be free, and the residual amino group may affect carthamin

unfavourably, because carthamin is usually unstable in the presence of basic compounds (SAITO et al. 1992).

Starch is not so effective stabilizer as Avicel cellulose and chitin (net retention 13.3 %). This may result from the fact that the straight chain of α-1,4 glucan and the branched chain of α-1,6 glucan in the macromolecule are not suitable for the stabilization of carthamin. It is possible that the α-configuration and/or the branched chain of starch and Sephadex G-15 prevents carthamin adsorption through steric hindrance. The negative results from observation on agar, xylan, hyaluronic acid and alginic acid support this, indicating that glucosyl units with β-configuration are indispensable for the adsorption and/or stabilization of carthamin.

In order to realize the existence of a chemical bonding mechanism between carthamin and cellulose, we conducted FT-IR spectral analyses. The data of the spectral measurement (Fig. 3) show a sharp band in the region about 1 600 cm^{-1}. Carthamin alone gives no such characteristic absorption band in the region. No corresponding signal is detectable in plain Avicel cellulose. This pronounced signal indicates the possibility that the frequency ν (>C=O) of carthamin contributes to a pair with the ν (OH) of the alcoholic hydroxil of each glucose unit on the cellulose molecule, presumably through a OH liner H-bond (>C=O·····H–O·····) (WEIDEMANN and ZUNDEL 1970, RASTOGI and ZUNDEL 1981, RASTOGI et al. 1981). Other minors signals at 1500–1350 cm^{-1} are supposed as well to be given by the chemical interaction between carthamin and Avicel cellulose, where additional intermolecular vibrations (including symmetrical and/or degenerate) of other carbonyl groups on carthamin and hydroxyls of glucose units of cellulose could occur (see arrows annexed in Fig. 3).

In conclusion, we summarize our evidence for a possible mechanism of carthamin binding on cellulose and related substances to express a stable red colouration. These are mainly as follows: (1) temperature, pH, buffer systems and buffer concentration affect carthamin adsorption towards cellulose, (2) molecular mass and its form influence the adsorption and stabilization of carthamin, (3) glucosyl units with β-configuration are essential, (4) alcoholic hydroxyl attached to the C-6 glucose units plays a leading role.

Based on the current data (SAITO and FUKUSHIMA 1986, SAITO 1990), a hypothetical formula is presented below (Fig. 4), indicating that the royal red colour of carthamin is preserved through a chemical binding mechanism (presumably hydrogen bonding(s)) between carthamin and polyglucoses. This is provided from the fact that carthamin can be recovered with organic reagents known as hydrogen bond splitting solvents. Cellobiose is not effective for retaining carthamin red colour, in particular at a high temperature (see Tab. 2), and cellulose completely loses its affinity for carthamin when it is treated with cellulase from *Aspergillus niger* (SAITO and FUKUSHIMA 1986).

No evidence for the integral site(s) of carthamin which would directly take part in the chemical bonding has been provided here. However, we can picture some of the specific chromophor groups of carthamin from its chemical structure and other data (TAKAHASHI et al. 1982). A carbonyl at C-2 of carthamin seems to

be the most important site to endow the pigment with its chromospecific character, because safflor yellow B, a bichalcone glycoside synonymous with carthamin, whose carbonyl at C-2 is reduced to a hydroxyl yielding two symmetric chalcoquinoides, is orange-yellow in colour (TAKAHASHI et al. 1984). To establish stable binding, another carbonyl (perhaps at C-1" position on the side phenyl straight chain on carthamin) is used to pair with hydrogen on the hydroxyl of glucosyl polymers. Through this process, a regulatory mechanism for specific red colour preservation (SAITO EFFECT) is introduced to the carthamin adsorbed macromolecules. Recent studies on the ultrastructure of cellulose have supported the co-existence of hydrogen bonds and van der Waals forces within glucan minisheet of dye-altered cellulose (COUSINS and BROWN 1997).

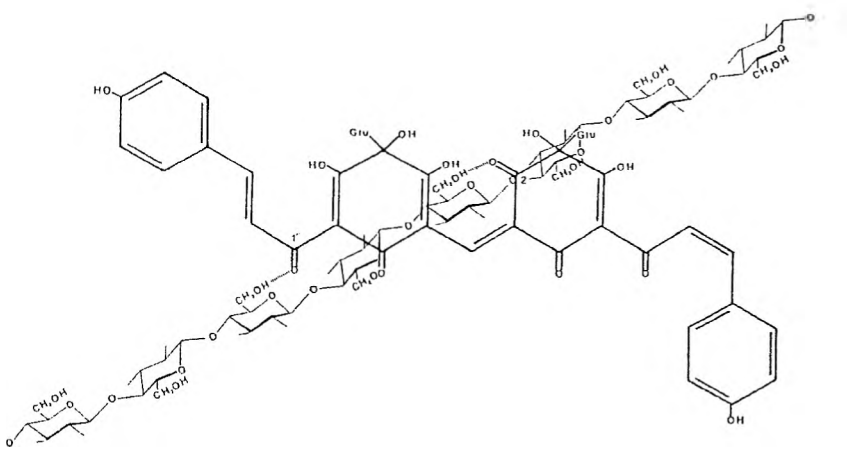

Fig. 4. A hypothetical scheme for the stable binding formation of carthamin with cellulose. To visualize the affinity sites on carthamin for cellulose, carthamin structure is drawn a little large. C-2 and C-1" are the proposed binding groups of carthamin; both of them could form a hydrogen bonding pair with primary alcoholic hydroxyls on glucose units of cellulose.

Acknowledgement

The author thanks S. TANI for technical assistance.

References

COUSINS, S. K., BROWN B. M., 1997: X-ray diffraction and ultrastructural analyses of dye-altered celluloses support van der Waals forces as the initial step in cellulose crystallization. Polymer 38, 897–902.

HAVLIKOVÁ, L., MIKOVÁ, K., 1987: Transformations of elderberry anthocyanins. Z. Lebensm. Unters. Forsch. 184, 289–293.

KANEHIRA, T., NARUSE, A., FUKUSHIMA A., SAITO K., 1990: Decomposition of carthamin in aqueous solutions: influence of temperature, pH, light, buffer

systems, external gas phases, metal ions, and certain chemicals. Z. Lebensm. Unters. Forsch. 190, 299–305.

RASTOGI, P.P., ZUNDEL, G., 1981: Aspartic acid-aspartate and glutamic acid-glutamate hydrogen bonds having great proton polarizability – IR investigation. Z. Naturforsch. 36c, 961–963.

RASTOGI, P.P., KRISTOF, W., ZUNDEL, G., 1981: Easily polarizable proton transfer hydrogen bonds between the side chains of histidine and carboxylic acid groups of glutamic and aspartic acid residues in proteins. Int. J. Biol. Macromol. 3, 154–158.

SAITO, K., FUKUSHIMA, A., 1986: Effect of external conditions on the stability of enzymically synthesized carthamin. Acta Soc. Bot. Pol. 55, 639–651.

SAITO, K., FUKUSHIMA, A., 1987: Effect of exogenous substances on carthamin red coloration. Food Chem. 26, 125–138.

SAITO, K., 1990: Possible locality of affinitive site(s) on cellulose for preservation of stable red colour of carthamin. Food Chem. 36, 243–251.

SAITO, K., FUKUSHIMA, A., 1991: Thermal stability of carthamin bound to insoluble polymers – effect of pH and organic and inorganic compounds. Int. J. Food Sci. Technol. 26, 21–26.

SAITO, K., YAMAMOTO, T., MIYAMOTO K.-I., 1992: Isolation and partial purification of carthamine: an instrumentation manual. Z. Lebensm. Unters. Forsch. 195, 550–554.

SAITO, K., MIYAMOTO K.-I., KATSUKURA, M., 1993a: Influence of external additives on the preservation of carthamin red colour: an introductory test for utilizing carthamin as a herbal colorant of processed foods. Z. Lebensm. Unters. Forsch. 196, 259–260.

SAITO, K., MATSUMURA, M., TAKAHASHI, M., 1993b: The sensitivity of carthamin and safflor yellow B colours towards redox substances. Acta Soc. Bot. Pol. 62, 25–31.

SAITO, K., MORI, T., 1994a: Colour stability of carthamin under alkaline conditions. Food Chem. 51, 105–107.

SAITO, K., MORI, T., 1994b: Restoration of the red colour of photo-bleached carthamine in calcium-alginate gel membranes. Food Res. Int. 27, 571–574.

SAITO, K., MORI, T., 1994c: Colour stability of carthamin dissolved in aqueous solutions with various pH values. Biologia (Bratislava) 49, 881–885.

SAITO, K., MORI, T., MIYAMOTO, K.-I., 1994: Stability of carthamin in calcium alginate beads. Food Chem. 50, 311–312.

SAITO, K., MURATA, T., MORI, T., 1995: Encapsulation of carthamin and safflor yellow B in calcium alginate beads – technical stabilization of the colours. Int. J. Food Sci. Technol. 29, 715–719.

TAKAHASHI, Y., MIYASAKA, T., TASAKA, S., MIURA, I., URANO, S., IKURA, M., HIKICHI, K., MATSUMOTO, T., WADA, M., 1982: Constitution of two colouring matters in the flower petals of *Carthamus tinctorius* L. Tetrahedron Lett. 23, 5163–5166.

TAKAHASHI, Y., SAITO, K., YANAGIYA, M., IKURA, M., HIKICHI, K., MATSUMOTO, T., WADA, M., 1984: Chemical constitution of safflor yellow B, a quinochal-

cone C-glycoside from flower petals of *Carthamus tinctorius* L. Tetrahedron Lett. 25, 2471–2474.

WEIDEMANN, E. G., ZUNDEL, G., 1970: Field-dependent mechanism of anomalous proton conductivity and the polarisability of hydrogen bonds with tunneling protons. Z. Naturforsch. 25A, 627–634.

Corresponding author:
Koshi Saito
Saito Phyto–Science Institute,
Biochemical Laboratory, Phyto-Dye Division,
No. 13-1007, N-7, W-12, Chuo-Ku,
Sapporo 060-0007,
Japan