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Possible Locality of Affinitive Site(s) on Cellulose for Preservation of Stable Red Colour of Carthamin

Koshi Saito

Department of Bioscience and Technology, School of Engineering,
Hokkaido Tokai University, Sapporo 005, Japan

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ABSTRACT

To search for the specific binding site(s) of cellulose to form a stable coloured complex with carthamin, interaction between carthamin and four different glucosyl polymers was studied as a function of pigment adsorbability to respective test polymers in an acidic buffer solution. A possible action site in the interaction was suggested to be primary alcoholic hydroxyl group(s) on the glucose units of the macromolecules examined. An equivalent amount of carthamin was fixed with cellulose and the resultant carthamin–cellulose complex subjected to spectroscopic analyses. In the complex, there were characteristic changes in the spectrophotometric patterns in the regions of 1630 cm^{-1} (infra-red) and 2260 nm (near infra-red), indicating that hydrogen bonding(s) exists between carthamin and cellulose. The above results are finally discussed in relation to the stable red colour expression of carthamin on cellulose.

INTRODUCTION

In a previous paper, we have demonstrated that cellulose is one of the most effective polymers for preserving carthamin red colour from its inevitable denaturation in aqueous media (Saito & Fukushima, 1986). Subsequently, the mechanism through which the stable coloration is maintained has been examined by using ionically charged or uncharged cellulose derivatives and it has been suggested that hydrogen bonding must play an important role in sustaining the red colour on cellulose (Saito & Fukushima, 1988).

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A number of phenolic compounds, including chalcoquinoids, are known to bind easily with both synthetic and natural macromolecular substances through hydrogen bonding (Loomis & Battaile, 1966; Lam & Shaw, 1970; Fukushima *et al.*, 1987; Saito & Fukushima, 1989). Whether or not the binding mechanism always contributes to the colour preservation is unclear.

Carthamin is one of the chalcoquinoidal pigments which can be divided into three building sub-blocks, C-glucopyranosyl, *p*-hydroxycinnamoyl and chalcoquinoidal residues; the latter two are bridged by an olephinic bond (Takahashi *et al.*, 1982). It is, therefore, possible to retain proton accepting groups in the molecule.

Our present interests are concerned with the recently observed carthamin stabilization and its control mechanism. Thus, the current study was planned to identify the affinitive site(s) on cellulose, which controls carthamin red coloration. Two studies, namely, comparison of affinitive binding capacities of polyglucoses and a spectroscopic survey of the possible binding mechanism, will be carried out in these experiments.

ABBREVIATIONS

CM, carboxymethyl; IR, infra-red; NIR, near infra-red.

MATERIALS AND METHODS

Materials

Carthamin (from *Carthamus tinctorius* L.), used throughout the experiments, was from our laboratory collection, which was purified to a microcrystalline state through successive column chromatographies and subsequent recrystallization (Saito *et al.*, 1983*a*,*b*). Cellulose powder was purchased from Macherey Nagel (Doren, FRG). CM-cellulose was provided by Seikagaku Kogyo Co., Ltd (Tokyo, Japan). Xylan (from *Betula platyphylla* Sukatchev var. *Japonica* (Miq.) Hara) was a gift. Other chemicals and reagents used were obtained from several commercial sources.

Conditioning of glucosyl polymers

An appropriate weight of each polymer was suspended in 200 mM NaOH and left overnight at room temperature (22–24°C) by stirring vigorously with a magnetic stirrer. After discarding the supernatant, the pellets were washed exhaustively with sufficient volume of deionized–

distilled water until no trace of Na^+ was detected. The pellets were transferred to 200 mM HCl and washings were carried out as done in alkaline treatment. The resultant acid-free polymers were dried in air and stocked in a desiccator over silica gel.

Affinity test of carthamin for insoluble polyglucoses

A known weight of each polymer was suspended separately in 50 mM citrate–phosphate buffer (pH 5·5) containing 55 μM carthamin and stirred for 10 s on a mixer. The mixture was passed through a glassfibre filter and the filtrate applied immediately for estimation of the binding rate of the pigment. The rate was determined by computing the amount of carthamin remaining in the filtrate.

Recovery of carthamin from glucosyl polymers

A given amount of the red-coloured polymers was placed on a Büchner funnel bearing a glassfibre filter and then 5 ml of aqueous acetone (60% by vol) was poured dropwise over each polymer. The amount of the pigment recovered was estimated by computing the data from measurement of the absorbance at 521 nm with a Shimadzu, type 150-02 spectrophotometer.

Preparation of carthamin–cellulose complex for spectroscopic measurement

Carthamin (5 mg) was dissolved in 100 ml of 50 mM citrate–phosphate buffer, pH 5·5, (55 μM carthamin). To this solution, 50 mg cellulose powder was suspended and the suspension shaken gently for 10 min at 22–24°C in an incubator (Yamato, type BT-46). The reddened powder was transferred to a Büchner funnel and washed three times with a sufficient volume of deionized–distilled water. The damp cellulose was then dried in an automatic air-circulation oven (Mitamura, type 1037) at 80°C for 24–48 h and the resulting dried matter was kept in a desiccator over silica gel before use.

Measurement of carthamin–cellulose complex by IR and NIR spectroscopies

IR-spectra were measured in micro-KBr disks using a Degilab, model FT-15E spectrophotometer, whose resolving power is 8 cm^{-1}. The scanning frequences were 100 and reference was air. KBr disks prepared were (in 200 mg KBr, each): 0·5 mg carthamin, 2·1 mg cellulose, 2·7 mg carthamin + cellulose. NIR-spectra were recorded by reflectance with a slit width of 40 nm on the micro-KBr pellets, used for all FT-IR spectral

measurements. A Japan Spectroscopic apparatus, model UVIDEC-590 fitted with a type of TIS-257 integration lamp, was used by applying MgO as a standard plate.

RESULTS AND DISCUSSION

For testing the specific absorbability of carthamin, four typical glucosyl polymers, whose terminal groups on the structural units were substituted variously, were selected and each polymer was treated in an acidic buffer solution containing a given concentration of carthamin. The carthamin amounts taken up by the test polymers are listed in Table 1 as micromolar concentrations per milligram of polymer. Cellulose powder shows the highest affinity for carthamin, as suggested in our previous papers (Saito & Fukushima, 1986; Saito & Fukushima, 1988). Chitosan comes next. CM-cellulose follows this, while xylan has the lowest affinitive capacity among the four. The ratios calculated from the table were comparable with each other as follows: 14·5:8·5:2·1:1·0. The differences in the carthamin absorbability originate solely from the constitution of the structural units in each polymer, for the recovery rate of fixed carthamin was not so varied as considered previously. The rate was computed as follows (%): cellulose powder (68·3), chitosan (67·5), CM-cellulose (45·6) and xylan (37·6). This relatively similar rate seems to be indicative of the possibility that the carthamin attracting forces in the test polymers are controlled by the same affinitive mechanism. The recovery ratios were compared to be; cellulose powder:chitosan:CM-cellulose:xylan = 1·8:1·8:1·2:1·0, respectively.

In Fig. 1, IR-spectra registered before or after fixation of carthamin to cellulose are presented. The results show that, when carthamin is fixed with cellulose, the spectral pattern ranging from 1100 to 1630 cm^{-1} changes clearly compared with that of carthamin alone. This spectroscopic modification can also be seen in NIR-spectra taken by a surface reflection monitoring technique using a carthamin-cellulose micro pellet (Fig. 2). In

TABLE 1
Adsorption of Carthamin to Polyglucose Derivatives

Polymer	*Polymer used (mg)*	*Carthamin adsorbed (μM/mg cellulose)*	*Adsorption rate (%)*
Cellulose powder	17	4·49	100
Chitosan	24	2·64	58·8
CM-cellulose	85	0·65	14·5
Xylan	50	0·31	6·9

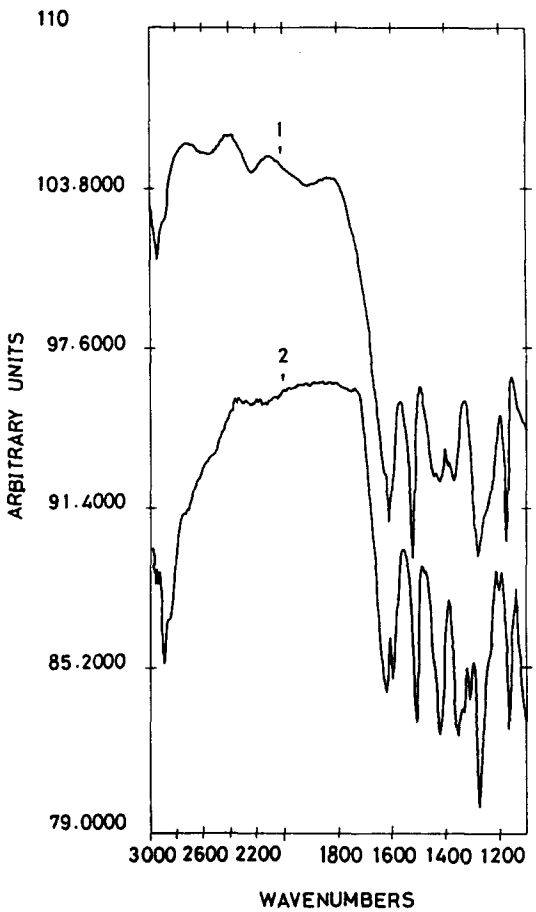

Fig. 1. IR-spectra of carthamin before or after adsorption to cellulose powder. 1, carthamin; 2, difference spectrum of carthamin adsorbed to cellulose and cellulose.

these spectra the characteristic light absorption appears at 2230 nm as seen in the figure. These two data are all suggestive of the binding mechanism of cellulose to carthamin or vice versa, possibly through hydrogen bonding. As noted above, carthamin is greatly stabilized on cellulose after being adsorbed by the polymer in various acidic solutions (Saito & Fukushima, 1986; Saito & Fukushima, 1988). The fundamentals of the carthamin stabilization seem to be sustained principally through a chemisorption which involves, essentially, the formation of a relatively weak chemical bond between the sorbing molecule and the surface of the adsorbent.

Based on our recent studies, we have deduced that a hydrogen bond must play an integral role in the carthamin red colour preservation (Saito & Fukushima, 1988). Our previous assumption is based on the following: (1) carthamin adsorption to cellulose is highly specific; (2) fixation of carthamin

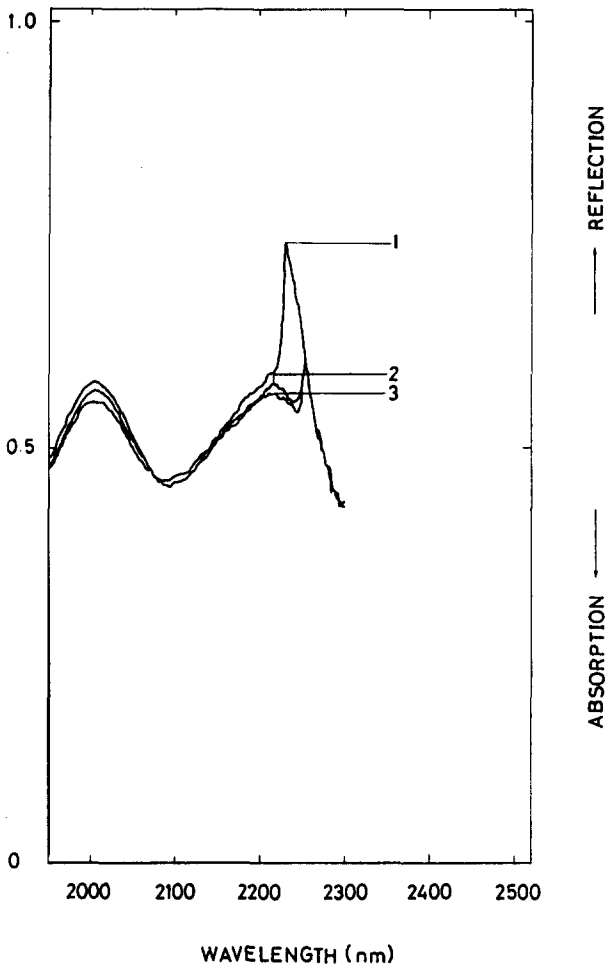

Fig. 2. NIR-spectra of carthamin before or after adsorption to cellulose powder. 1, cellulose; 2, carthamin–cellulose complex; 3, carthamin.

is possible over a wide range of temperatures and (3) fixed carthamin can be recovered easily by using hydrogen bond dissociation reagents.

For locating the carthamin attracting site(s), we have used four typical non-branched polyglucoses, among which two have free primary alcoholic hydroxyl groups and the other two lack them or they are partially deficient through chemical substitution. The results obtained in the present experiments provide relatively clear indication about the specificity of attracting carthamin to test polymers; one type comprises cellulose powder and chitosan, and the other covers CM-cellulose and xylan. Cellulose powder attracts carthamin from acidic solutions in the most specific way. It carries one primary alcoholic hydroxyl on each structural building block.

Chitosan is a useful carthamin adsorbent, though the efficiency is inferior to cellulose. This compound is constructed of deacylated 2-amino D-glucose residues, polymerized linearly by β-1,4 linkages and it also has the primary alcoholic hydroxyls. CM-cellulose shows a lower affinitive capacity for the pigment, as observed previously (Saito & Fukushima, 1988). This is one of the glycollic acid derivatives and the terminal hydroxyls are substituted by acetyl groups; as a consequence, its primary hydroxyls are practically free. The exhibited low affinity presumably results from its chemical modification. A markedly characteristic adsorbability is presented by xylan. It fixes the pigment at an extremely low level (7%) (see Table 1). The compound is one of the pentosan derivatives, which are long, linear β-1,4-xylopyranosyl polymers and, of course deficient in alcoholic hydroxyls, especially in the primary position of the building unit. The above evidence obviously suggests that carthamin is entrapped, in the main, by polymers at the primary alcoholic hydroxyls.

An alternative way by which a molecule might achieve stability is to form a cross-linked structure with another molecule through a physical and/or chemical binding mechanism. The hydrogen bond is able to create a large number and wide variety of structures via this specific binding process, leading to the formation of multiple stable macro-molecules. It has been reported that hydrogen bonds show great proton polarizabilities when double minimum proton potentials or potentials with broad flat wells are present in these hydrogen bonds (Weidemann & Zundel, 1970; Janoscheck *et al.*, 1972; Rastogi & Zundel, 1981*a*) and that the polarizabilities arise in various homoconjugated and heteroconjugated bonds between side chains of proteins (Rastogi *et al.*, 1980, 1981; Kristof & Zundel, 1980*a*,*b*; Rastogi & Zundel, 1981*b*). The large proton polarizabilities can usually be visualized in IR- and NIR-spectra as characteristic signals resulting from continuous intense light absorptions. The IR-spectra (Fig. 1) indicate that carthamin alone shows no characteristic band in the region at about 1600 cm^{-1}. On the other hand, when carthamin is fixed with cellulose, a new small but sharp band appears in the region at 1630 cm^{-1}, indicating that the frequency ν(C=O) of the chalcoquinoid contributes to a pair with the ν(OH) of the alcoholic hydroxyl of each glucose unit in the cellulose molecule, presumably through an OH linear H-bond ($\rangle$C=O$\cdots$H—O—$\cdots$). This suggestion is supported by the data from NIR spectral analysis. An intense light absorption occurs at about 2200 nm in the cellulose–carthamin complex (see Fig. 2), which is also indicative of a close interaction between cellulose and carthamin via the hydrogen bonding process.

In this study we provide no information about affinitive position(s) in the carthamin molecule. The amount of carthamin fixed on cellulose is too small (approximately 0·6%) to obtain fully resolved spectroscopic patterns.

Elaborate techniques, provided by more sensitive apparatus, will probably execute this difficult problem in the near future.

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