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Spectrophotometric Evidence for the Solubilization Site of Betalain Pigments in Membrane Biomimetic Systems

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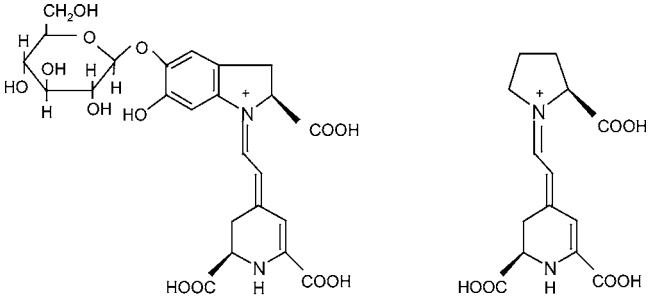
The solubilization site of two betalain pigments, namely, betanin and indicaxantin, into L-α-dipalmitoylphosphatidylcholine (DPPC) and dimyristoylphosphatidylcholine (DMPC) vesicles was investigated by a spectrophotometric study. Pigment absorbance was monitored by varying phospholipid concentration, at a constant temperature that was varied in a range including the main phase transition temperature (T_m) of the relevant phospholipid bilayer. Maximum betanin absorption increased with the increase of DPPC concentration within the entire temperature range, reaching a plateau. The binding constant (K_b) of the pigment, calculated according to a pseudo-two-phase model, varied with the temperature, indicating that betanin is located at the hydrophobic interior of the bilayer. Other measurements of binding of betanin to DMPC and of indicaxanthin to either DPPC or DMPC vesicles ruled out that these compounds were solubilized in the hydrophobic interior of these bilayers.

KEYWORDS: Betalain pigments; vesicles; bio-mimetic membranes

INTRODUCTION

Betalain pigments are condensation products of betalamic acid with various amino acids to form betaxanthins or with *cyclo*-DOPA or glycosyl derivatives of *cyclo*-DOPA to form betacyanins (*1*, *2*). These molecules are synthesized and stored in the vacuolar compartment of plants in the order *Cariophyllales* (*3*, *4*), where they are usually dissolved as bis-anions (*5*). Two of these compounds, betanin and indicaxanthin (**Figure 1**), that characterize the fruits of cactus species such as *Opuntia ficus indica* (*6*) have received recent attention for their antioxidant activity in a number of biological lipid environments in vitro, from human low density lipoproteins to cell membranes (*7*−*9*). The interaction of the molecules with the lipid structures has been considered at the basis of such an activity. Dialysis experiments provided evidence that betanin (*7*) and indicaxanthin (*10*) can bind to biological membranes (*7*) and to large unilamellar DPPC or soybean-PC liposomes (*10*). Charge-related interactions with polar headgroups of membrane constituents, or even inclusion or adsorption reactions with the lipid aggregates, could be accounted for by the structure of these molecules that indeed may be considered as amphyphilic-like compounds (**Figure 1**).

Some insight into the interaction of betanin and indicaxanthin with lipid moieties including biomembranes can be obtained from partition studies of these molecules between a water pseudo-phase and a vesicular bilayer of known phospholipid composition. Since vesicles are thermodynamically and kinetically stable and easily reproducible, they provide easier access for exploiting various techniques in solutions and suspensions than the biological interfaces.



Betanin Indicaxanthin

Figure 1. Molecular structure of betanin and indicaxanthin. Experimental evidence (*7*, *10*) suggests that the molecules are amphipatic.

A bilayer composed of only one kind of phospholipid undergoes well-defined temperature-dependent chemicophysical alterations, the most prominent being observed at the main phase transition temperature (T_m), at which the bilayer changes from a gel-like state to a fluid one, the so-called crystal−liquid phase. When vesicles of a surfactant whose T_m is known are used, changing the vesicular fluidity by varying the temperature may be a suitable strategy to investigate the solubilization site of compounds bound to the bilayer. The characteristic absorbance bands of betanin and indicaxanthin, with absorbance peaks at 536 and 482 nm, respectively (*1*), and their changes as a function

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of temperature and vesicle concentrations were used in this study as tools to investigate solubilization and the location of pigments in a lipid bilayer of either L-α-dipalmitoyl-phosphatidylcholine (DPPC) or dimyristoyl-phosphatidylcholine (DMPC).

EXPERIMENTAL PROCEDURES

Materials. DMPC and DPPC were purchased from Avanti Lipids and used without further purification. Betanin and indicaxantin were obtained from local cactus pear fruits (*O. ficus indica*). Briefly, the phytochemicals were separated from a methanol extract of the pulp by liquid chromatography on Sephadex G-25 (*11*). Fractions containing the pigment were submitted to cryo-dessiccation, then resuspended in PBS and submitted to a solid-phase extraction according to Stintzing et al. (*3*). The dessiccated material was finally resuspended in 1% acetic acid in water and submitted to semipreparative HPLC using a Varian Pursuit C18 column (250 mm × 10 mm i.d.; 5 μm; Varian) and eluted with a 20 min linear gradient elution from solvent A (1% acetic acid in water) to 20% solvent B (1% acetic acid in acetonitrile) with a flow of 3 mL/min. Spectrophotometric revelation was at 482 and 536 nm for indicaxanthin and betanin, respectively. The elution volumes relevant to the two phytochemicals were collected. Samples after cryo-dessiccation were stored at −80 °C. The purified phytochemicals were suspended in PBS, and the concentration of the samples was evaluated spectrophotometrically in a DU-640 Beckman spectrophotometer by using a molar coefficient at 482 nm of 42 800 and at 536 nm of 65 000 for indicaxanthin and betanin, respectively (*1*).

All solutions were prepared by using deionized water obtained with a Culligan Pharma System 20.

Preparation of Vesicles. Stock aqueous vesicular dispersions have been prepared by sonication of lipid suspensions in 5 mM phosphate buffered saline (PBS), pH 7.4, by a titanium probe connected to a 100 W Pabish high intensity ultrasonic processor, using 5 s pulses of 60 W, with a 1 s interval, for 30 min. The temperature was kept constant at 55 and 37 °C for DPPC and DMPC, respectively. Samples were subsequently filtrated using Millex-HV filters (PDVF) with a pore diameter of 45 μm to remove eventual titanium particles. Vesicle dispersions at the required lipid concentration were obtained by diluting the stock dispersion with PBS and were used immediately. By this method, small unilamellar vesicles were obtained, whose size was independent of time for at least 3 h (*12*, *13*), with aqueous dispersions maintaining a clear aspect and no turbidity from adhesion or fusion processes evident. When required, either betanin or indicaxanthin was added to the vesicular preparations as PBS solutions, to obtain a 2.0×10^{-6} mol dm^{-3} final concentration. Under these conditions, the addition of the pigments did not change the ionic strength of the medium, nor did it cause osmotic effects at the vesicles, so that the vesicular stability was not affected.

Spectrophotometry. All measurements were performed with a Beckman DU-640 spectrophotometer, equipped with thermostatted compartments for 1.00 cm cuvettes and appropriate magnetic stirring devices. The spectrophotometer was interfaced to a computer for both data collection and analysis. The temperature control was obtained by a thermostat Heto Therm ±0.1 °C. Spectra of betanin and indicaxanthin were monitored between 350 and 700 nm, either in the absence or in the presence of variable vesicle concentrations, at a constant temperature that was varied in the range of 25.0−37.0 and 35.0−48.0 °C for DMPC and DPPC vescicles, respectively. The choice of these temperatures was dictated from the knowledge that the main transition temperature of the chosen phospholipids (*14*) lies within the studied range, so that measurements were carried out both in the gel-like vesicle state at $T < T_m$ and in the liquid-crystal-like one at $T > T_m$. All the pigment spectra were reproducible within the sensibility of the spectrophotometer.

RESULTS AND DISCUSSION

In this work, the partition of betanin and indicaxanthin between two pseudo-phases, aqueous and vesicular, formed by phospholipids of different acyl chain lengths (i.e., DPPC and DMPC) was investigated by spectrophotometric measurements to provide information about the interaction of these pigments with a membrane bio-mimetic system.

Betalains are heat-sensitive (*4*, *15*, *16*). The influence of either DPPC or DMPC vesicles on the stability of betanin and indicaxanthin was preliminarily checked. Spectra of a PBS solution of both pigments were monitored between 350 and 750 nm, in the range of 25.0−48.0 °C, at 6 min time intervals, for 60 min. No significant variation of the spectrum, neither in the band shape nor in the intensity, was observed between 25.0 and 35.0 °C, whereas the maximum absorbance progressively decreased within the observation time, at temperatures higher than 40.0 °C, although the profile of the spectrum remained the same. **Figure 2** shows typical spectral variations of betanin and indicaxanthin at 25.0 °C (**Figure 2A**,**C**) and 48.0 °C (**Figure 2B**,**D**), while the loss of absorbance on varying the temperature is shown in **Figure 3**. The spectra of betanin and indicaxanthin, in the presence of variable concentrations of either DMPC or DPPC vesicles, were then monitored for 60 min at a constant temperature that varied between 25.0 and 37.0 °C and between 35.0 and 48.0 °C for DMPC and DPPC vesicles, respectively, and compared with the relevant spectra in the absence of phospholipids. At any given temperature, the absorbance kinetics and the shape of the spectra were not affected by the presence of vesicles (not shown), suggesting that neither betanin nor indicaxanthin undergo a chemical reaction with the phospholipids.

The maximum absorbance of betanin varied as a function of DPPC concentration, indicating that the chromophoric center of the molecule was sensitive to its molecular environment, and as a function of temperature. The analysis carried out between 35.0 and 48.0 °C showed that the absorbance of betanin at 536 nm increased at the increase of DPPC in the range of 0.05−1.0 mM, tending to a plateau at the highest concentrations. **Figure 4** reports a typical absorbance versus [DPPC] curve measured at 36.0 °C. Similar plots were observed within the entire temperature range.

The spectrophotometric data were analyzed according to a pseudo-two-phase distribution model. In accordance, the pigment is distributed between the vesicular pseudo-phase (v) and the aqueous one (w) (*17*), according to the following equilibrium:

$$\text{betanin}_v \rightleftarrows \text{betanin}_w$$

with a partition coefficient $P = [\text{betanin}]_v/[\text{betanin}]_w$ and a binding constant K_b given by

$$K_b = \frac{P}{[\text{DPPC}]} \tag{1}$$

that expresses the affinity of betanin for the vesicular pseudo-phase. An implication of this model is that the absorbance of the sample (A) results from the sum of the absorbance of the pigment in the aqueous pseudo-phase (A_w) and of that in the vesicular one (A_v). Then, A will depend on the DPPC concentration according to

$$A = A_w + \frac{(A_v - A_w)K_b[\text{DPPC}]}{1 + K_b[\text{DPPC}]} \tag{2}$$

A nonlinear least-squares analysis of the spectrophotometric data by means of eq 2 allowed us to calculate the binding constant of betanin, which varied as a function of temperature (**Figure 5**).

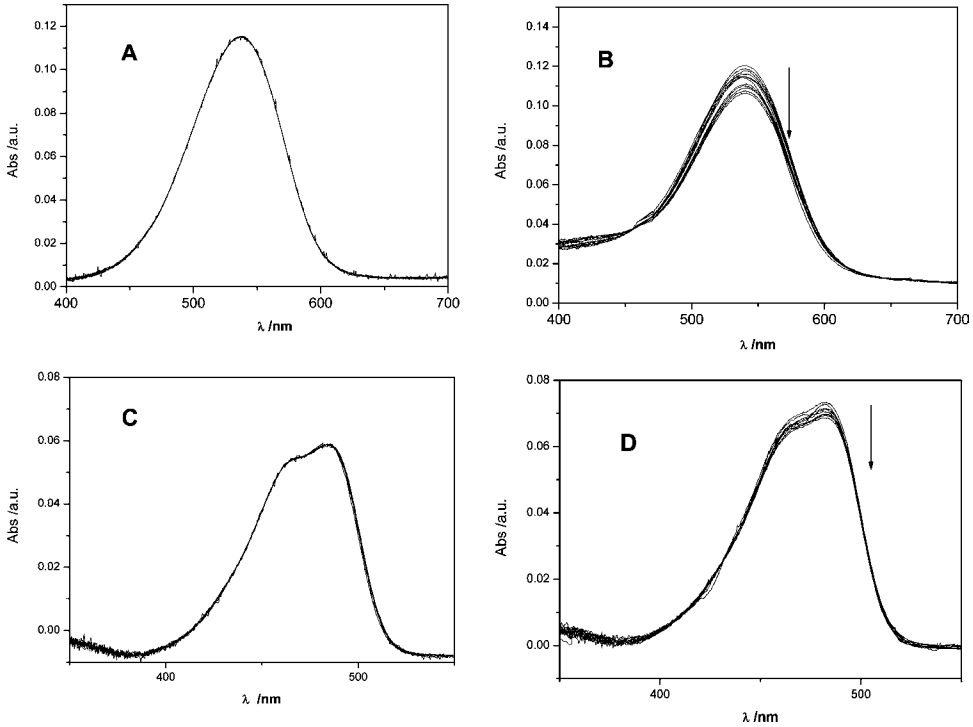

Figure 2. Typical spectra of 2.0×10^{-6} mol dm^{-3} betanin (**A** and **B**) and 2.0×10^{-6} mol dm^{-3} indicaxantin (**C** and **D**) in PBS. (**A** and **C**) Recordings at $t = 25.0$ °C for 60 min and (**B** and **D**) recordings at $t = 48.0$ °C for 60 min.

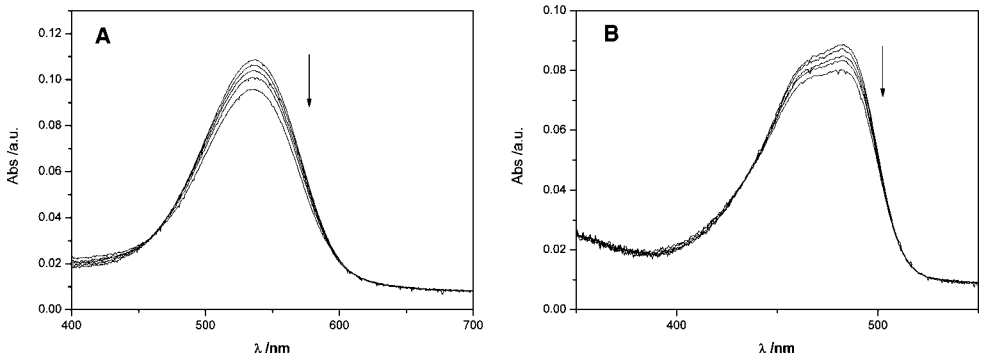

Figure 3. Typical UV–vis spectra of 2.0×10^{-6} mol dm^{-3} betanin (**A**) and indicaxantin (**B**), after a 60 min incubation in PBS at different temperatures, in the range of 25.0–48.0 °C.

An important property that distinguishes the vesicles from other aggregated systems is the tuneable and temperature-dependent fluidity of the bilayer. Below the T_m, the acyl chains of phospholipids are in a rigid conformational state that permits a highly ordered, strongly hydrophobic gel-like arrangement of the bilayer. The increase of temperature favors rapid thermal movements of the fatty acyl chains, resulting in a progressively less rigid arrangement of the phospholipids so that, at the T_m, the bilayer changes to a fluid crystal−liquid state. Variation of fluidity may, or may not, affect the binding of a species with a lipid bilayer, which can provide information about the solubilization loci of the species (i.e., the hydrophobic core or the polar palisade domain). When the binding of betanin to the DPPC vesicles was investigated as a function of temperature, the chosen range (35.0−48.0 °C) included the T_m of DPPC (41.3 °C), in order that measurements were performed in both the gel-like state and the liquid−crystal-like one. Remarkably, the binding affinity of betanin to DPPC vesicles showed a bimodal dependence on the temperature. In the range of 35.0−43.0 °C, the calculated constants decreased with an increase of temperature, and then, after the inflection point above the phase transition temperature, a striking increase of the values was observed in the range of 43.0−48.0 °C (**Figure 5**). The trend of K_b below the T_m shows that betanin experiences an environment of hydrophobicity that depends on temperature changes and encounters a progressive loss of interaction due to the increased permeability of the vesicular double layer. Since modifications of both fluidity and permeability are associated to conformational surfactant chain changes on increasing the temperature, we may conclude that betanin is solubilized at the hydrophobic interior of the vesicular bilayer (*14*, *18*), otherwise a net independence of the binding constant on temperature had to be found (*19*). This has indeed been demonstrated for species incorporated in a DPPC bilayer in a region between the hydrophilic wet headgroups and the hydrophobic part, the so-called palisade domain (*14*).

The variation of the binding constant above the T_m has to be rationalized by a molecular variation of the pigment at increasing temperatures. According to our measurements, betanin underwent a time-dependent decrease of the maximum absorbance, with no change of λ_{max}, in the range of 37.0−48.0 °C (**Figure 3A**). A number of studies showed that decarboxylation of betanin can occur upon heating, with production of a less polar species that maintains the chromophoric group of the

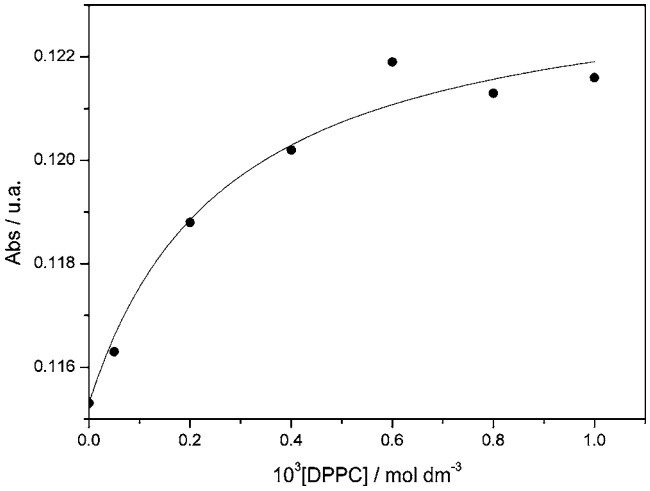

Figure 4. Absorbance of 2.0×10^{-6} mol dm^{-3} betanin (monitored at 536 nm) as a function of DPPC concentration at pH = 7.4, $t = 36.0$ °C. (●) Experimental data and (—) curve calculated by a nonlinear least-squares fit to eq 3.

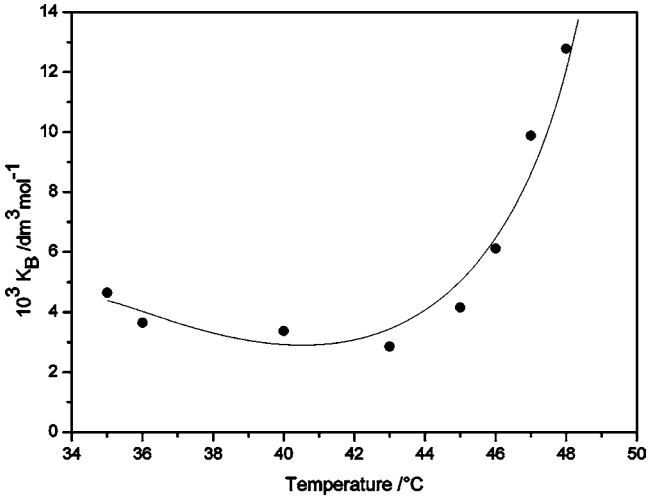

Figure 5. Temperature dependence of the binding constant K_b of betanin to DPPC vesicles. ($T_m = 41.3$ °C for an aqueous dispersion of DPPC vesicles).

parent compound, and shows a batochromic shift of λ_{max} at 533 nm (*20*−*22*). A temperature-dependent decarboxylation of betanin to some extent may be hypothesized under our conditions, which could account for the observed variations of K_b.

As has been previously observed (*14*), molecules like alcohol or metal porphyrins, interacting with a lipid bilayer, may modify the fluidity (i.e., the characteristic T_m). It is worth noting that the temperature at which the K_b trend shows the discontinuity point is slightly higher than the T_m of DPPC vesicles. This would confirm that betanin interacts with the lipid domain and reduces the mobility of the fatty acyl chains, thereby increasing the microviscosity in the interior of the bilayer. Moreover, the smooth variation of the binding constant with the temperature suggests that there must be a coexistence between the gel-like and the liquid−crystal-like phases near the transition temperature. This is consistent with a location of betanin in the hydrophobic core of the bilayer. The domain of coexistence is indeed caused by the incorporation of additives in the phospholipid bilayers (*14*, *18*, *23*), which contributes to disturbing the orientation of the lipids within the bilayer.

To obtain further information on the interactions of betanin with phospholipid bilayers, the spectrophotometric study of solubilization was extended to the vesicles of DMPC, a surfactant with a hydrophobic tail shorter than DPPC by two CH_2 groups. The lack of changes in the spectrophotometric recordings with varying either lipid concentration or temperature did not allow us to make a conclusion about the solubilization of betanin in DMPC bilayers. The spectral evidence rules out that betanin is located in the vesicular hydrophobic core but cannot assess whether it is located near the polar headgroup in a very hydrophilic region or if it is confined in the aqueous phase. However, the interaction of betanin with the hydrophobic interior of a DPPC, but not DMPC, vesicle would indicate that the length of the acyl chain is a crucial factor to allow us to determine the location of betanin.

Attempts to ascertain the solubilization of indicaxanthin in either DPPC or DMPC vesicles were inconclusive. Indeed, changes in the maximum absorbance of indicaxanthin with varying either lipid concentration or temperature were not observed, which allows us only to exclude that indicaxanthin is located in the hydrophobic core of both vesicles. When considering previous findings from dialysis experiments showing an affinity of indicaxanthin for unilamellar DPPC liposomes (*10*), a location of the pigment in the palisade domain of this bilayer could be hypothesized. The lower hydrophobicity of indicaxanthin with respect to betanin and/or the length of the structure with respect to palmitic acid seems to favor other kinds of interactions and would account for such a location.

In spite of the observed binding to either natural membranes (*7*) or liposomes (*10*), betalamic acid derived pigments, either betacyanins or betaxanthins, are known to be highly solubile in water. Our present data with DPPC vesicles suggest a location of betanin, but not indicaxanthin, at the hydrophobic interior of the bilayer, showing some peculiar chemicophysical aspects of these molecules. When considering the structural arrangement of the two compounds, interactions of the betalamic acid portion, which is common to the two molecules, with the bilayer core seem to be less feasible. Rather, under the conditions of our experiments, the N of betalamic acid and the two carboxyl groups may have an affinity for the polar headgroups of phosphatidylcholine. It can be hypothesized that the phenol moiety of *cyclo*-DOPA of betanin may be involved in hydrophobic binding, despite the presence and vicinity of the sugar moiety. A relatively higher hydrophobicity of betanin, with respect to indicaxanthin, may also be deduced from the retention times, with indicaxanthin eluting earlier than betanin in a HPLC reversed-phase system (*3*). At any instance, the length of the fatty acid chain should be crucial for the proper orientation of the molecule.

Biological membranes are composed of a mixture of phospholipids; however, they are always maintained in the fluid state (i.e., above the T_m) at 37.0 °C. In light of the potential beneficial effects of betalains in vivo (*24*, *25*), the present findings stimulate further studies to provide a deeper knowledge about interactions of betalain pigments with cellular lipid structures.

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