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Inhibitory Effect of Cyclodextrins on the Discoloration Reaction of an Anthocyanidin, Pelargonidin Chloride, in Acidic Media

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Abstract. An anthocyanidin, pelargonidin (PG), loses its color with time in acidic media. The rate determining step of the discoloration reaction at pH 1–4 is the nucleophilic attack of the OH^- ion on PG to give a hemiacetal, which readily isomerizes to the corresponding α-diketone. Native, branched, and methylated cyclodextrins (CDs) form inclusion complexes with PG to retard the discoloration. The inhibitory effect (copigmentation) of CDs on the PG discoloration is slight in α-CD, significant in β- and γ-CDs, and the largest in heptakis(2,6-dimethyl)-β-CD (DM-β-CD). The β-CD and DM-β-CD include the phenyl moiety of PG, whereas γ-CD includes the benzopyrylium moiety of PG. The CD cavities protect the reaction site of included PG from the attack of the OH^- ion.

Key words: cyclodextrin, pelargonidin, anthocyanin, discoloration, inclusion complex, copigmentation, NMR spectroscopy.

1. Introduction

Anthocyanin pigments (glycosylated polyhydroxy derivatives of 2-phenylbenzopyrylium or flavylium salts) are universal in flowers and fruits [1]. The color of anthocyanin is stable in plant cells but unstable after extraction from the cells [2]. Extracted anthocyanin quickly loses color in an acidic aqueous solution by the addition of the OH^- ion, or H_2O followed by loss of a proton (H_2O/-H^+), to its chromophore (the flavylium cation, A^+) to give colorless hemiacetal (B) and chalcone (C) according to the reaction scheme shown in Figure 1 for the simplest anthocyanin, callistephin (R = glucoside). The discoloration reaction of A^+ is completely retarded in plant cells by the formation of a supramolecular assembly between various components of the pigment and copigments (copigmentation) [2, 3].

Cyclodextrin (CD) forms inclusion complexes with a variety of organic molecules to give supramolecules. Thus, it might be anticipated that anthocyanin is stabilized by complexation with CD. However, the few articles thus far reported

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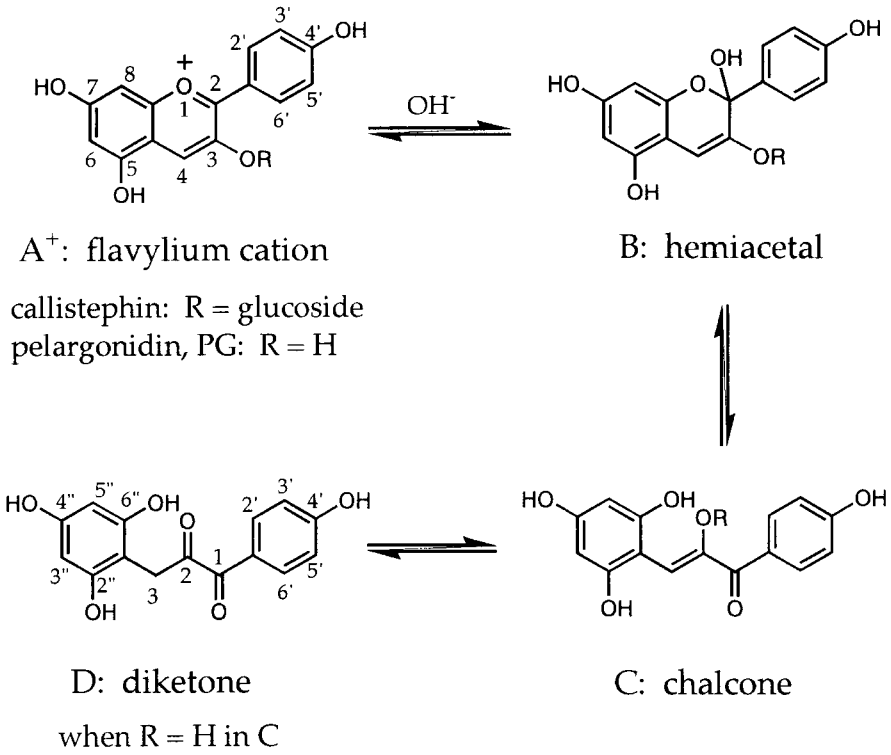

Figure 1. A reaction scheme for the discoloration reactions of an anthocyanin, callistephin (R = glucoside), and pelargonidin (PG, R = H) in acidic media.

[4–6] revealed that the complexation of anthocyanins with β-CD promotes the discoloration (anti-copigmentation) in acidic media.

The present work deals with the effect of various CDs on the discoloration of pelargonidin (PG in Figure 1), which is an aglycone (anthocyanidin) of anthocyanin. As far as we are aware, no work has been reported on the interactions between CD and anthocyanidins. Interestingly, CDs did not promote but retarded the discoloration (copigmentation) of PG in acidic media. Hence, the molecular mechanism of the inhibitory effect of CDs was examined in detail.

2. Experimental

2.1. MATERIALS

The PG chloride was purchased from Sigma Chemical Co. and used without further purification. The α-, β-, and γ-CDs, 6-*O*-α-D-glucosyl-β-CD (G1-β-CD), and 6-*O*-α-maltosyl-β-CD (G2-β-CD) were supplied by Ensuiko Seito Co. Ltd. The hexakis(2,6-dimethyl)-α-CD (DM-α-CD), heptakis(2,6-dimethyl)-β-CD (DM-β-CD), and heptakis-(2,3,6-trimethyl)-β-CD (TM-β-CD) were of

reagent grade and commercially available. The D_2O (Isotec, 99.9%), DCl (Wako, 37%), sodium 3-trimethylsilyl-1-propanesulfonate (TSS, Merck), and dimethyl sulfoxide-d_6 (DMSO-d_6, Isotec, 99.9%) were of reagent grade and used for ^{1}H NMR measurements.

2.2. Apparatus

The absorption spectra were measured by a Shimadzu Model UV-2100 spectrophotometer. The sample solutions were kept at 298 K by means of a constant temperature bath. The ^{1}H NMR spectra were recorded using a JEOL Model JNM-A400 FT NMR spectrometer (400 MHz) at 298 K, unless otherwise noted. A D_2O solution of 5 mmol dm^{-3} TSS was loaded into a glass capillary of 1.0 mm diameter and used as an external reference. The phase-sensitive ROESY (rotating frame nuclear Overhauser enhancement spectroscopy) spectra of PG complexes with CDs were acquired with a mixing time of 1.0 s, 512 points for t_2, and 256 points for t_1 followed by zero-filling.

2.3. Kinetics

A weighed amount of PG chloride was dissolved in ethanol to give a stock solution of 1.0 mmol dm^{-3} PG. Various amounts of CD were dissolved in aqueous buffer solutions mainly composed of citric acid and disodium hydrogenphosphate. An aliquot (0.04 mL) of the PG stock solution was added to a buffer solution (2.00 mL) in a quartz cell (1.0 cm), which was previously maintained at 298 K. Changes in absorption spectra with time were recorded in a range of 700–200 nm. The first order rate constants (k_1) were determined by the curve-fitting analysis of changes in absorbance at a constant wavelength with time.

2.4. Identification of reaction product

The discoloration reaction of PG was also followed by means of ^{1}H NMR spectroscopy in a 2 : 8 (v/v) mixture of DMSO-d_6 and H_2O containing 0.10 mol dm^{-3} HCl at 293 K. The HCl was added to retard the discoloration reaction sufficiently to follow a change in the ^{1}H NMR spectrum of PG with time. The DMSO-d_6 was used for dissolving PG in H_2O sufficiently for the measurement of the ^{1}H NMR spectrum, as well as for the deuterium lock of the spectrometer. H_2O was used in place of D_2O, since rapid H/D exchange reactions were found for the C(4)-, C(6)-, and C(8)-Hs of PG, together with the corresponding protons of the reaction product, in a 2 : 8 (v/v) mixture of DMSO-d_6 and D_2O containing 0.10 mol dm^{-3} DCl at 293 K. The PG solution just after preparation gave ^{1}H NMR signals at δ = 8.10 [s, 1H, C(4)-H], δ = 8.03 [d, 2H, J = 8.8 Hz, C(2′, 6′)-H], δ = 6.71 [d, 2H, J = 8.5 Hz, C(3′, 5′)-H], δ = 6.52 [s, 1H, C(8)-H], and δ = 6.37 [s, 1H, C(6)-H]. Upon standing for a day at 293 K, the PG solution gave new ^{1}H NMR

signals which were attributed to 1-(4-hydroxyphenyl)-3-(2,4,6-trihydroxyphenyl)-1,2-propanedione (compound D in Figure 1): δ = 7.81 [d, 2H, J = 8.5 Hz, C(2′, 6′)-H], δ = 6.96 [d, 2H, J = 8.5 Hz, C(3′, 5′)-H], δ = 5.94 [s, 2H, C(3″, 5″)-H], and δ = 3.97 [s, 2H, C(3)-H]. Similar ^{1}H NMR signals were also observed for a PG solution at pH 3.0 upon measurement ca. one hour after preparation of the solution.

3. Results and Discussion

3.1. Discoloration reaction of PG in the absence of CD

Figure 2 illustrates the change in the absorption spectrum of PG with time in a buffer solution at pH 2.91 and 298 K. Absorption maxima (λ_{max}) were observed at ca. 505, 420, and 265 nm. The absorbances for these maxima decreased, whereas that around 300 nm increased, with time. Clear isosbestic points were found at ca. 350 and 283 nm during the change, indicating that a simple reaction from a reactant to a product is accompanied by the discoloration. The decrease in absorbance at λ = 505 nm followed first-order kinetics. The first-order rate constant (k_1) was determined by the curve-fitting analysis of the change. Similar experiments were carried out for PG in buffer solutions in a range of pH values from 1–8. Figure 3 illustrates changes in λ_{max} of initial PG solutions and log k_1 with pH. The λ_{max} of PG at 505 nm was unchanged in buffer solutions at pH values lower than 4.5, indicating that PG is in a form of the flavylium cation in this pH range. A marked bathochromic shift was found in the λ_{max} at pH values higher than 4.8, suggesting that a part of the PG hydroxyl groups dissociates to give the corresponding quinoidal bases [2]. The log k_1 value linearly increased with pH in a pH range of 1–4, reached a maximum at pH ca. 4.3, and then decreased. The slope of the plot in a pH range of 1–4 was ca. 1.0, indicating that the discoloration reaction is catalyzed by the OH^- ion, or H_2O followed by loss of a proton (H_2O/-H^+) as suggested by Brouillard [2]. Thus, it is reasonable to assume that the rate determining step of the discoloration reaction is the addition of the OH^- ion to the flavylium cation of PG to give the corresponding hemiacetal.

The main product of the discoloration reaction was identified by ^{1}H NMR spectroscopy to be the α-diketone D shown in Figure 1. The diketone structure of the product was specified on the basis of the appearance of a singlet ^{1}H NMR signal at δ = 3.97, assigned to the aliphatic C(3)-Hs of D, in place of the disappearance of a singlet signal due to the aromatic C(4)-H of PG at δ = 8.10. The cleavage at the O(1)-C(2) bond of PG was confirmed by the appearance of a singlet signal at δ = 5.94 due to the magnetically equivalent C(3″)- and C(5″)-Hs of D in place of the disappearance of singlet signals at δ = 6.52 and 6.37 due to the C(8)- and C(6)-Hs of PG, respectively. The α-diketone D is a tautomer of the corresponding 2-hydroxychalcone C which is, in turn, a product of a ring-opening reaction of the hemiacetal B in Figure 1. The keto-enol equilibrium between C and D will lie so far to the keto side that no such intermediates as B and C are detected by ^{1}H NMR spectroscopy during the discoloration reaction of PG.

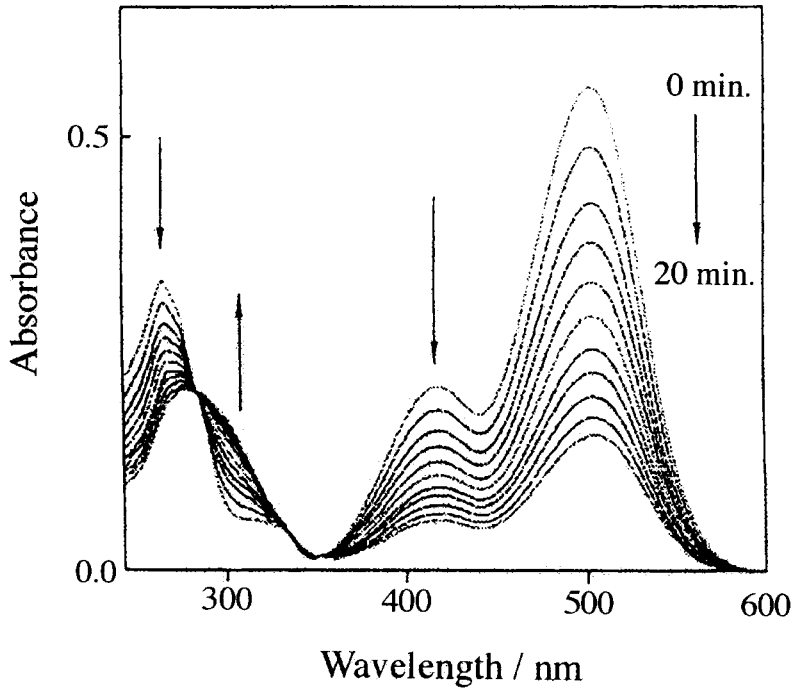

Figure 2. Changes in the absorption spectrum of PG with time in a buffer solution at pH 2.91 and 298 K.

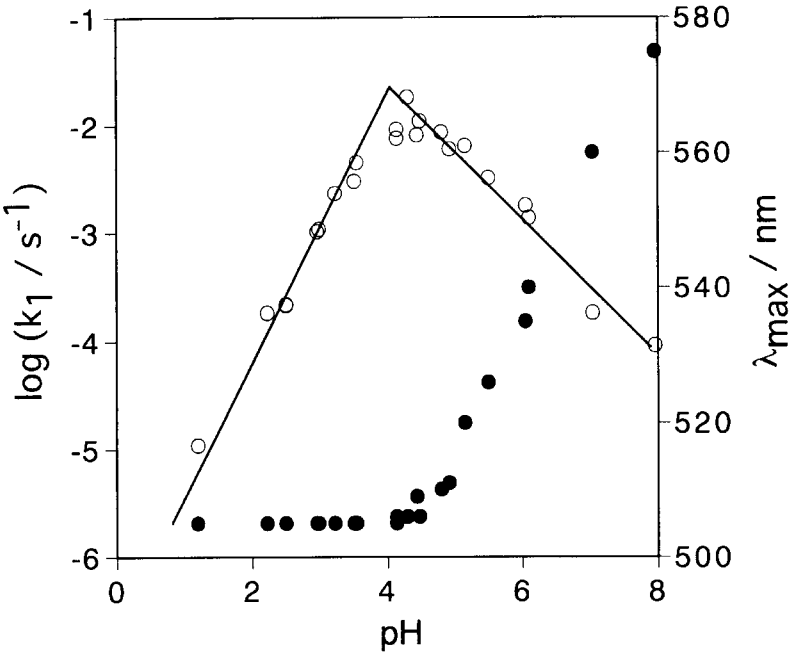

Figure 3. Plots of log (k_1/s^{-1}) (○) and λ_{max}/nm (●) vs. pH for PG solutions at 298 K.

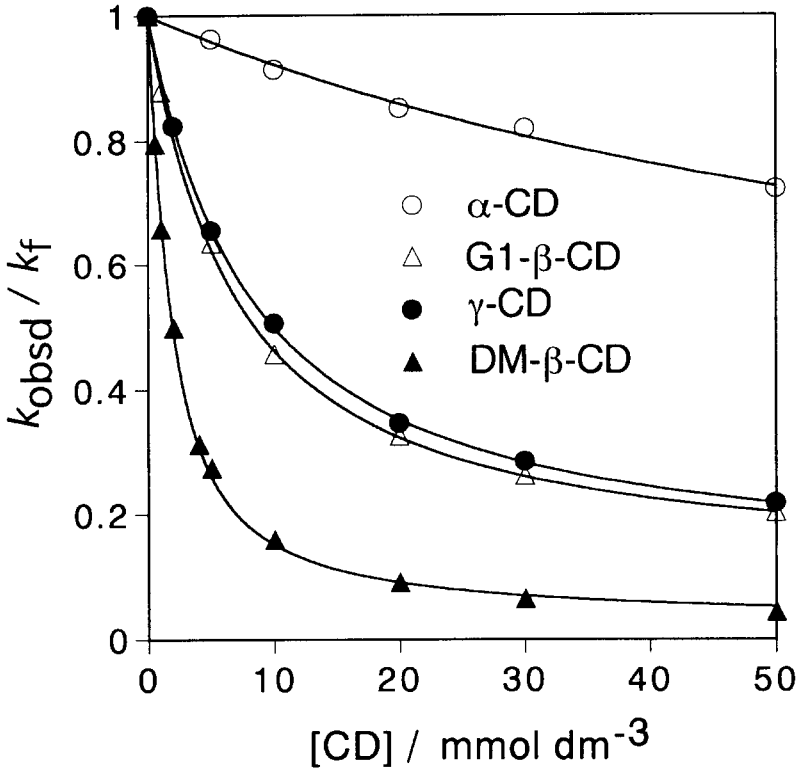

Figure 4. Plots of k_{obsd}/k_f vs. [CD]/mmol dm^{-3} for some typical CDs in a buffer solution at pH 3.0 and 298 K.

3.2. Effect of CD on the discoloration reaction of PG

The effect of various CDs, including native, branched, and methylated CDs, on the rate of PG discoloration was examined in a buffer solution (pH 3.0) at 298 K. In general, the first-order rate constants (k_{obsd}) for the discoloration of PG in the presence of CDs were smaller than those (k_f) in the absence of CD and decreased with increasing CD concentration ([CD]). Figure 4 illustrates the plots of k_{obsd}/k_f vs. [CD] for some typical CDs. Upon assuming 1 : 1 complexation between PG and CDs, k_{obsd} is represented by

$$k_{\text{obsd}} = k_f x_f + k_c x_c, \tag{1}$$

where k_c is the first-order rate constants for the complexed PG, x_f and x_c, the mole fractions of free and complexed PG, respectively. Since $x_f + x_c = 1$, we obtain

$$k_{\text{obsd}}/k_f = 1 - (1 - k_c/k_f)x_c. \tag{2}$$

The value of k_c and the binding constant (K_a) for a 1 : 1 complex of PG with CDs were determined by a non-linear curve-fitting analysis of changes in k_{obsd}/k_f

Table I. The first-order rate constants of discoloration (k_c) and binding constants (K_a) for PG complexes with various CDs at pH 3.0 and 298 K.

	$10^5 k_c/s^{-1}$	k_c/k_f^a	$K_a/mol^{-1}\ dm^3$
α-CD	27.3	0.261	12
β-CD	5.2	0.049	109
γ-CD	9.4	0.090	143
G1-β-CD	9.2	0.087	120
G2-β-CD	6.8	0.065	111
DM-α-CD	18.0	0.170	40
DM-β-CD	0.3	0.003	530
TM-β-CD	21.2	0.199	53

[a] The k_f value for the free PG is equal to 105×10^{-5} s^{-1}.

with [CD]. The calculated curves (solid lines in Figure 4) were well-fitted to the observed data, indicating that the assumption of 1 : 1 complexation is valid. The obtained k_c and K_a values are summarized in Table I.

The k_c value was the largest, and the K_a value was the smallest, for α-CD among the examined CDs. This means that the inhibitory effect of α-CD on PG discoloration is the smallest. The inhibitory effect of DM-α-CD was also small, compared with the β-CD analog. The dimensions of the phenyl and benzopyrylium moieties of PG were estimated by use of the CPK model to be 0.55 and 0.67 nm, respectively. On the other hand, the diameters of α-, β-, and γ-CDs have been evaluated to be 0.47–0.52, 0.60–0.64, and 0.75–0.83 nm, respectively [7]. Thus, the interior cavities of α-CD and DM-α-CD will be too small to deeply accommodate the phenyl or benzopyrylium moiety of PG. The inhibitory effects of native β- and γ-CDs, as well as those of branched β-CDs, were significantly larger, indicating that these CDs deeply include the phenyl or benzopyrylium moiety of PG to retard the discoloration of PG. The largest inhibitory effect was observed for DM-β-CD, in which the k_c and K_a values were ca. 17 times smaller and 5 times larger than those for native β-CD, respectively. The interior cavity of DM-β-CD is deeper and more hydrophobic than native β-CD, so that the former will be more advantageous for the protection of PG from a discoloration reaction than the latter. In contrast to DM-β-CD, TM-β-CD showed only a small inhibitory effect. It is known that the macrocyclic ring of TM-β-CD is remarkably distorted by the steric hindrance arising from methyl groups attached to O(3), whereas that of DM-β-CD has a round shape [8, 9]. The distortion in the TM-β-CD macrocycle may be disadvantageous both for complexation with PG and for the protection of PG from a discoloration reaction.

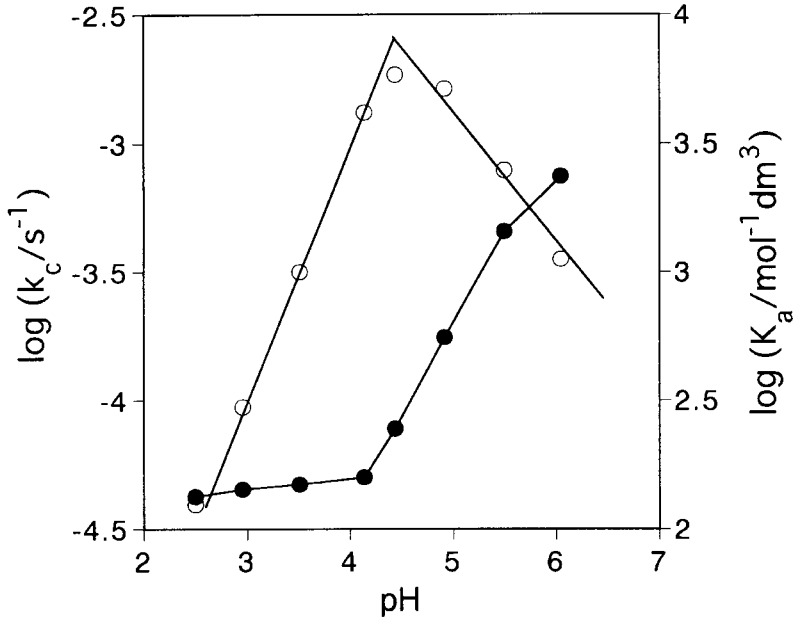

Figure 5. Plots of log (k_c/s^{-1}) (○) and log (K_a/mol^{-1} dm^3) (●) vs. pH for PG complexes with γ-CD at 298 K.

How is the discoloration of PG retarded by complexation with CD? A possible mechanism is that PG is bound to CD in the form of the flavylium cation and is protected from the nucleophilic attack of the OH^- ion by the CD cavity. Another possible mechanism is that CD preferentially includes the quinoidal bases of PG which are neutral molecules and less reactive to the OH^- ion than the flavylium cation. In order to judge which is the case, changes in k_c and K_a with pH were examined on a γ-CD-PG system (Figure 5). The log k_c value linearly increased with pH in a pH range of 1–4, reached a maximum at pH ca. 4.1, and then decreased. The slope of the plot in a pH range of 1–4 was equal to ca. 1.0, indicating that the discoloration reaction of complexed PG is catalyzed by the OH^- ion, similarly to that of free PG. On the other hand, the K_a value was virtually constant in a pH range of 1–4 and rapidly increased with increasing pH higher than 4.2. A marked bathochromic shift of λ_{max} was also observed for the absorption spectra of PG solutions containing γ-CD at pH values above 4.2. These facts show that PG included within the γ-CD cavity is in the form of the flavylium cation at pH lower than 4.0. At higher pH, complexation of the quinoidal bases of PG with γ-CD will begin to occur. The k_c and K_a values for the quinoidal PG will be smaller and larger, respectively, than those for the flavylium PG. Hence, we judged that the protection of the flavylium cation from the attack of the OH^- ion by CD complexation is responsible for the inhibitory effect of CDs on PG discoloration in an aqueous solution at pH 1–4.

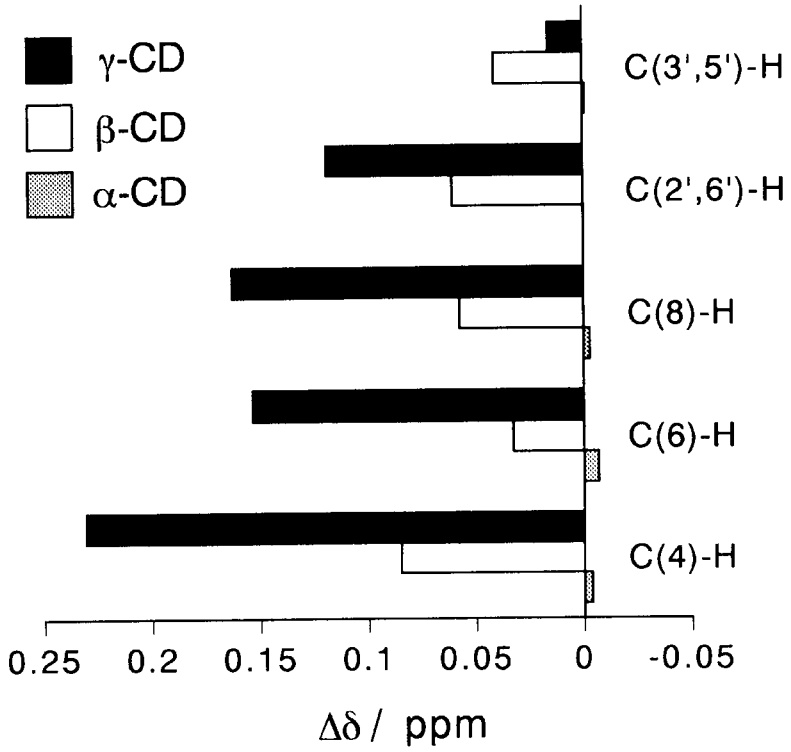

Figure 6. Changes ($\Delta\delta$) in chemical shifts of PG protons with the addition of 13 mmol dm^{-3} α-, β-, and γ-CDs to a solution of 13 mmol dm^{-3} PG in 2 : 8 (v/v) DMSO-d_6-D_2O containing 0.10 mol dm^{-3} DCl at 298 K.

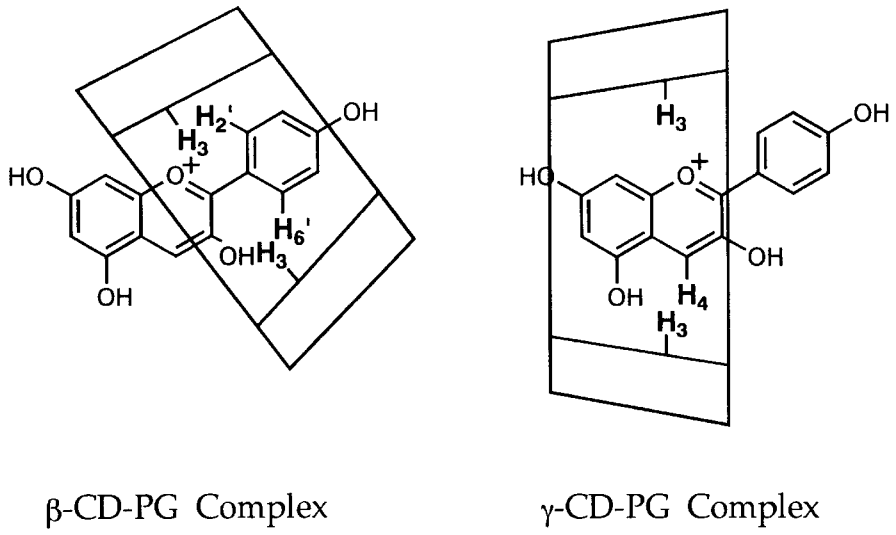

Figure 7. Possible molecular structures of PG complexes with β- and γ-CDs in acidic media.

The inhibitory effect of CDs on PG discoloration in acidic media is in contrast to the promoting effect of β-CD on the discoloration of callistephin (PG 3-glucoside) at pH 2 thus far reported [4–6]. The presence of 3-glucoside in callistephin inhibits the conversion of its chalcone C to D in Figure 1, since the chalcone is no longer in a labile enol form. Thus, the colored flavylium cation of callistephin is in equilibrium with the colorless forms such as hemiacetal and chalcone, and the absorption spectrum due to the flavylium cation of callistephin shows no change with time at pH 2. The addition of β-CD results in a marked decrease in absorbance of the cation. This process is reversible, and the effect of β-CD is attributed to a preferential inclusion of the colorless forms, rather than the colored flavylium cation, within the β-CD cavity. On the other hand, the chalcone from PG is in an enol form which readily converts to the corresponding colorless keto form D in Figure 1. This process is virtually irreversible. Thus, what we have observed is not an equilibrium process as in the case of callistephin but a kinetic process in which the rate determining step is the nucleophilic attack of the OH^- ion to the colored flavylium cation of PG. This will be a main reason why the effect of CD on PG discoloration is different from that on callistephin discoloration.

3.3. ^{1}H NMR SPECTROSCOPY OF CD COMPLEXES WITH PG

In order to obtain structural information on CD complexes with PG, ^{1}H NMR spectra were measured for PG, CDs (α-, β-, and γ-CDs), and their equimolar (13 mmol dm^{-3}) mixtures in solutions of 2 : 8 (v/v) DMSO-d_6-D_2O containing 0.10 mol dm^{-3} DCl at 298 K. The free PG gave ^{1}H NMR signals similar to those shown in the Experimental. Changes ($\Delta\delta$) in chemical shifts (δ) of PG protons with the addition of α-, β-, and γ-CDs are illustrated in Figure 6. α-CD gave only small $\Delta\delta$ values, probably due to very weak interactions with PG. β-, and γ-CDs caused significant downfield shifts in all the protons of PG. Among them, the C(4)-H of PG gave the largest $\Delta\delta$s in both β-, and γ-CDs. The second largest $\Delta\delta$ was observed for the C(2′,6′)-H of PG in β-CD and for the C(8)-H of PG in γ-CD, suggesting that β-, and γ-CDs include the phenyl and benzopyrylium moieties, respectively, of PG as illustrated in Figure 7. This presumption was substantiated by the measurements of phase-sensitive ROESY spectra for PG-CD systems. A clear cross peak was observed between the C(2′,6′)-H of PG and the C(3)-H of β-CD, indicating that the phenyl moiety of PG is included within the β-CD cavity. On the other hand, a clear cross peak appeared between the C(4)-H of PG and the C(3)-H of γ-CD, indicating that the benzopyrylium moiety of PG is included within the γ-CD cavity. In both cases, however, the reaction site C(2) of PG is protected by the CD cavities from the attack of the OH^- ion. The complexation of PG with β-, and γ-CDs also caused large upfield shifts in the C(3)- and C(5)-Hs of the CDs, which are located within the CD cavities. The diamagnetic shielding by the aromatic moieties of PG will be responsible for the large upfield shifts.

4. Conclusions

Pelargonidin (PG) lost its color with time in acidic media. The discoloration reaction was investigated by means of UV-visible absorption spectrophotometry and ^{1}H NMR spectroscopy. The logarithm of the first-order rate constant for the discoloration reaction increased in proportion to the solution pH in a range of 1–4, indicating that the rate determining step of the reaction was the nucleophilic attack of the OH^- ion to PG to give a hemiacetal, which readily isomerized to the corresponding α-diketone. The formation of the diketone was confirmed by ^{1}H NMR spectroscopy of the reaction product.

Native, branched, and methylated cyclodextrins (CDs) formed inclusion complexes with PG to retard the discoloration reaction of PG at pH 3.0. The inhibitory effect of CDs was slight in α-CD, DM-α-CD, and TM-β-CD, significant in β-CD, G1-β-CD, G2-β-CD and γ-CD, and the largest in DM-β-CD. The ROESY spectra of CD complexes with PG in a strongly acidic medium showed that β-CD includes the phenyl moiety of PG, whereas γ-CD includes the benzopyrylium moiety of PG. The inhibitory effect of CDs was attributed to the protection of the PG reactive site from the attack of the OH^- ion by the CD cavities. This so-called copigment effect of CDs was in contrast to the thus far reported anti-copigment effect of β-CD on callistephin (PG 3-glucoside).

At pHs higher than 4, some of the hydroxyl groups of PG dissociated to give the quinoidal bases, which were more resistant to discoloration and more strongly bound to γ-CD than the flavylium cation. The mechanism of discoloration of the quinoidal bases remains to be solved.

References

1. J. B. Harborne and R. J. Grayer: 'The Anthocyanins', in J. B. Harborne (ed.), *The Fravonoids*, pp. 1–20, Chapman and Hall (1988).
2. R. Brouillard: 'Flavonoids and Flower Colour', in J. B. Harborne (ed.), *The Fravonoids*, pp. 525–538, Chapman & Hall (1988).
3. T. Kondo, K. Yoshida, A. Nakagawa, T. Kawai, H. Tamura, and T. Goto: *Nature* **385**, 515 (1992).
4. T. Yamada, T. Komiya, and M. Akaki: *Agric. Biol. Chem.* **44**, 1411 (1980).
5. O. Dangles, M. C. Wigand, and R. Brouillard: *Phytochemistry* **31**, 3811 (1992).
6. O. Dangles, C. Stoeckel, M. C. Wigand, and R. Brouillard: *Tetrahedron Lett.* **33**, 5227 (1992).
7. W. Saenger: *Angew. Chem. Int. Ed. Engl.* **19**, 344 (1980).
8. K. Harata, K. Uekama, M. Otagiri, and F. Hirayama: *Bull. Chem. Soc. Jpn.* **56**, 1732 (1983).
9. K. Harata: *Chem. Lett.* **1984**, 1641.