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Components of protocyanin, a blue pigment from the blue flowers of *Centaurea cyanus*

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Abstract

The components involved in the formation of protocyanin, a stable blue complex pigment from the blue cornflower, *Centaurea cyanus*, were investigated. Reconstruction experiments using highly purified anthocyanin [centaurocyanin, cyanidin 3-*O*-(6-*O*-succinylglucoside)-5-*O*-glucoside], flavone glycoside [apigenin 7-*O*-glucuronide-4′-*O*-(6-*O*-malonylglucoside)] and metals, Fe and Mg, showed the presence of another factor essential for the formation of protocyanin. The unknown factor was revealed to be Ca. Reconstructed protocyanin using anthocyanin, flavone, Fe, Mg, and Ca was identical with protocyanin from nature in UV–Vis and CD spectra, and was isolated as crystals for the first time. In addition, substitution of the metal components in protocyanin with other metals was also examined.

Keywords: *Centaurea cyanus*; Compositae; Cornflower; Protocyanin; Blue pigment; Anthocyanin; Flavone glycoside

1. Introduction

Protocyanin, a blue pigment from the blue cornflower, *Centaurea cyanus*, was first isolated by Bayer (1958) and was described as a metal complex containing anthocyanin, polysaccharide, Fe and Al (Bayer et al., 1966). Hayashi et al. (1961) isolated it as crystals and demonstrated that protocyanin was a high molecular-weight organo-metallic compound containing Fe, Mg, anthocyanin, a flavonoid-like substance and carbohydrate (Saito et al., 1961; Saito and Hayashi, 1965), similar to the metallo-anthocyanin commelinin from the blue flower of *Commelina communis* (Hayashi et al., 1958; Mitsui et al., 1959). The anthocyanin in protocyanin had long been thought to be cyanidin 3-*O*-glucoside-5-*O*-glucoside (cyanin), (Willstätter and Everest, 1913), but it was determined to be cyanidin 3-*O*-(6-*O*-succinylglucoside)-5-*O*-glucoside, centaurocyanin (**1**) (Takeda and Tominaga, 1983; Tamura et al., 1983). On the other hand, the flavonoid-like substance was identified as apigenin 7-*O*-glucuronide-4′-*O*-(6-*O*-malonylglucoside) (**2**) (Tamura et al., 1983).

To clarify the nature of this complex pigment, a reconstruction experiment would be an important step as in the case of commelinin (Takeda and Hayashi, 1977; Takeda, 1977). In a similar manner as in commelinin, we had attempted to reconstruct protocyanin from the metal ions, Fe and Mg, and the organic components, **1**, **2**, which were prepared from purified protocyanin. However, such a stable blue complex pigment was not obtained. Meanwhile, Kondo et al. (1994) reported reconstruction of protocyanin using **1**, **2**, Fe and Mg, and presented a model structure of the pigment (Kondo et al., 1998).

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In this investigation, we found the presence of another factor required for the formation of protocyanin and succeeded in the reconstruction of the complex pigment and also in isolation of protocyanin as crystals for the first time, which would provide a powerful tool to characterize this complicated molecule by X-ray crystallography. In addition, substitution experiments of the metal components in protocyanin, which would be useful for further investigations, are also described.

2. Results and discussion

The blue pigment (ca. 100 mg) from the blue cornflower was dissolved in 1 ml of 5% formic acid. By the acid treatment, the blue color of the pigment gradually changed into red. The red reaction mixture was applied to Sephadex LH 20 column chromatography (28 × 400 mm) using H_2O as an eluant and fractions of 4.5 ml each were collected (nos. 1–130). Flavone **2** (Fig. 1) was contained in fraction nos. 50–74 and anthocyanin **1** in nos. 94–130. Reconstruction of the blue complex pigment was attempted using **1** and **2** above obtained together with metal ions, Fe^{2+} and Mg^{2+}. However, no stable complex pigment could be obtained. The results suggested that the presence of some other component was involved in the formation of the stable blue complex pigment. Subsequently, all other fractions were examined for the presence of the factor, by addition of the fractions to the mixtures of 1, 2, Fe and Mg. Fraction nos. 20–40 were found to contain the factor, as a blue complex pigment showing the same absorption spectrum as that of protocyanin was obtained. The fraction nos. 20–40 were collected and concentrated, and subjected to Sephadex LH 20 column chromatography once again. After evaporation to dryness, a small amount of colorless residue was obtained which had no absorption in the UV region. We estimated that the factor would be a high molecular compound as it eluted rapidly on Sephadex column chromatography. However, some attempts for further purification of the factor using column chromatography with Sephadex G-75 or G-100, HPLC with ODS and prep. TLC were unsuccessful because of loss of the activity. Finally, energy dispersive X-ray spectrometry of the residue indicated the presence of a considerable amount of metallic elements, that is, Mg, Ca, K and Na. The contents of the metallic elements of the sample were Mg 67.2%, Ca 21.9%, K 1.8% and Na 2.9%, respectively, in weight per cent. This fact suggested that the factor might be Ca. Indeed, a stable blue complex pigment was formed using **1**, **2**, Ca instead of the factor, Mg and Fe. Thus, the factor was suggested to be Ca.

The reason why the metal elements eluted rapidly through Sephadex column was unclear. However, the fractions might contain macromolecular substances such as polysaccharides having acidic residues.

For confirmation that Ca is an essential component of protocyanin, further experiments were carried out using purified **1** and **2**. In particular, **1** and **2** were passed

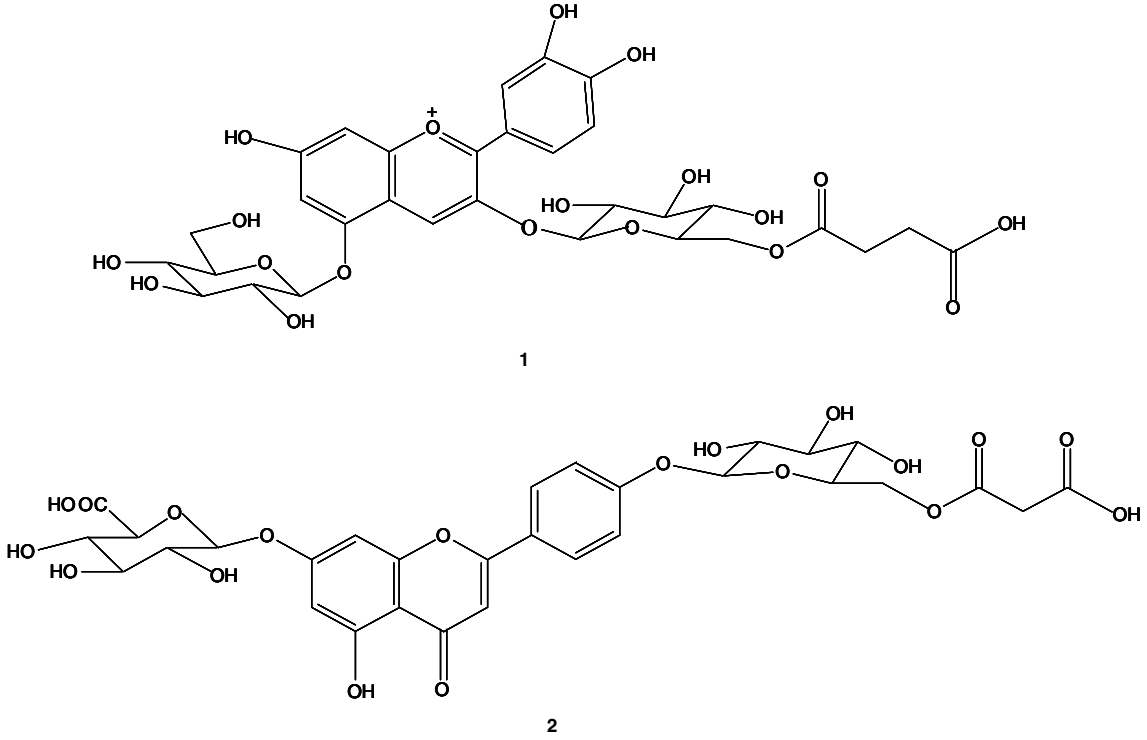

Fig. 1. The structures of anthocyanin **1** and flavone glycoside **2**.

Table 1
Formation of the blue complex pigment with anthocyanin (**1**), flavone glycoside (**2**), Fe^{2+}, Mg^{2+} in mole ratio of 1:1:0.1:2 and Ca^{2+} in various mole ratios

Mole ratio of Ca used	Blue pigment separable on Sephadex column	Amounts of the complex pigment formed (absorbance at 574 nm[a])
0	Trace	0.03
1	+	0.74
2	+	0.97
3	+	1.30
5	+	1.18
6	+	0.69

[a] Light path length of 3 mm.

through CM cellulose columns before use to prevent contamination of metal ions. Reconstruction of protocyanin was carried out as in the case of commelinin (Takeda et al., 1984). To the mixtures of **1**, **2**, Mg and Fe (1:1:0.1:2 in mole ratio), Ca was added in various mole ratios (0–6). After repeating evaporation of the solution and redissolving the residue in water, each mixture was passed through a Sephadex column using water as an eluant, whereby the protocyanin formed eluted rapidly and separated from other materials. As shown in Table 1, protocyanin was not formed without Ca, that is, protocyanin was not obtained from the mixtures containing only Fe and Mg as metals. However, the yield of protocyanin increased as the amount of Ca rose. At approximately 3 mole ratio of Ca, the amount of protocyanin formed reached a maximum and addition of excess Ca decreased the yield of the blue complex pigment. These results indicate that Ca is essential for the formation of protocyanin. As for the mole ratios of Fe and Mg added to the reaction mixtures, ratios of 0.1 for Fe and 2 for Mg were effective for the formation of the blue complex. In this investigation, Fe^{2+}, in the form of $Fe(SO_4)$, was used exclusively for providing the Fe. However, a blue complex pigment formed using Fe^{3+}, in the form of $FeNH_4(SO_4)_2$, showed an absorption spectrum identical to that of the above reconstructed protocyanin, as reported by Kondo et al. (1994).

For further characterization of the reconstructed protocyanin, the complex pigment was synthesized in quantity. After purification, the reconstructed protocyanin was obtained as crystals (Fig. 2). The pigment obtained was identical with protocyanin from nature in UV–Vis absorption spectra (λ_{max} 267, 317, 574 and 676 nm) and CD spectra ($\lambda_{vis\text{-}ext}$ 559, 600 and 639 nm) (Fig. 3), and also in mobility toward the anode using paper electrophoresis (36 mm, 1 h in 0.1 M acetate buffer of pH 4.80 on No. 50 filter paper, 400 V, 0.8 mA/cm) (Hayashi et al., 1961). The stability of the reconstructed protocyanin in aqueous solutions was almost the same as that of natural protocyanin as shown in Fig. 4.

Quantitative measurements of the components of the reconstructed and natural protocyanin were performed by the spectrophotometric method for **1** and **2**, and inductively coupled plasma (ICP) spectroscopy for metals. Ca is obviously contained in both pigments. The mole ratios of the components, **1**, **2**, Fe, Mg and Ca were approximately 6:6:1:(1–2):3 in the reconstructed protocyanin and 6:6:1:2:3 in natural protocyanin, respectively (Table 2).

To examine whether Ca can be substituted with other metals in the formation of the blue pigment, mixtures of **1**, **2**, Fe and Mg (1:1:0.1:2 in mole ratio) were added with the following metals (3 mole ratio) in the form of acetates, respectively: Mn, Co, Ni, Zn, Cd, Sr, Ba, Cu, K, Na and Ca as a control. As shown in Table 3, Ba and Sr formed stable blue complex pigments which showed

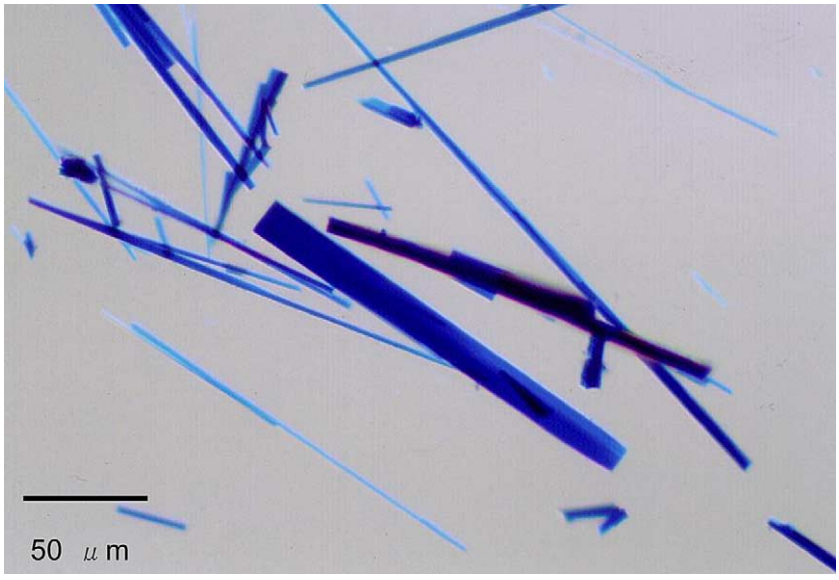

Fig. 2. Crystals of reconstructed protocyanin.

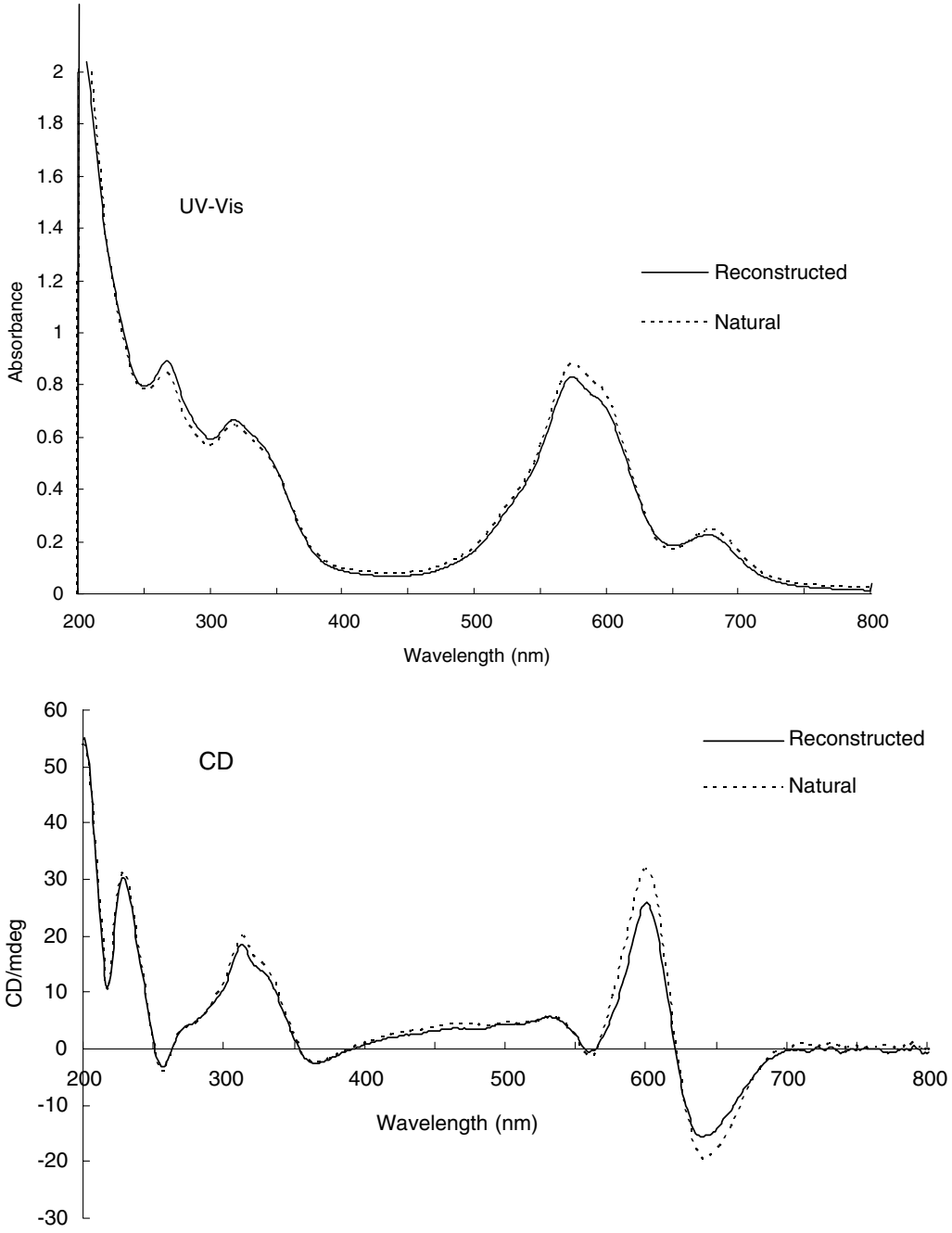

Fig. 3. UV–Vis and CD spectra of reconstructed and natural protocyanin in 0.05 M acetate buffer of pH 4.80 (light path length of 1 mm).

a practically identical pattern of absorption spectra with protocyanin. In addition, the complex pigments with Ba and Sr were more stable than that with Ca. Among other metals examined, Zn and Cd also formed small amounts of similar complex pigments which had two absorption maxima in the visible region, but these pigments were unstable. The results showed that Ca in protocyanin could be substituted with Ba or Sr, both also belonging to the alkaline earth metal group.

In the same manner as above, substitution of Mg in protocyanin with other metals was examined using mixtures of **1**, **2**, Fe and Ca (1:1:0.1:3 in mole ratio) and the following metal acetates (2 mole ratio), Mn, Co, Ni, Zn, Cd, Sr, Ba and Cu, respectively. As shown in Table 4, similar blue complex pigments were obtained with Mn, Co, Ni, Zn and Cd. Among them, the blue complex pigments formed with Mn and Cd showed practically the same pattern of absorption spectra with protocyanin. Substitution of Fe in protocyanin with Al^{3+} ($AlCl_3$) as well as the above other bivalent metals was also attempted, however, a blue pigment similar with protocyanin was not obtained without Fe.

In this investigation, we revealed the components of protocyanin to be **1**, **2**, Fe, Mg and Ca. Involvement

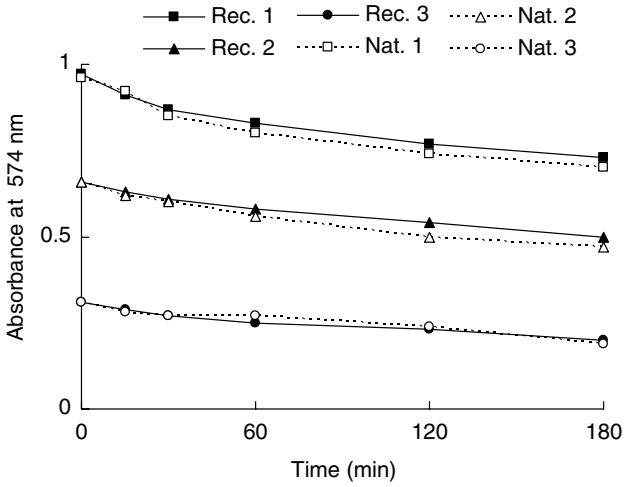

Fig. 4. Stability of reconstructed and natural protocyanin in three different concentrations (0.05 M acetate buffer of pH 4.80, light path length of 3 mm, 21 °C). Rec., reconstructed protocyanin; Nat., natural protocyanin. 1, 150 mg/l; 2, 115 mg/l; 3, 60 mg/l.

of Ca in the formation of protocyanin, as metal components together with Fe and Mg, was indicated by reconstruction experiments using highly purified materials, **1** and **2**. Kondo et al. (1998) demonstrated that protocyanin is a Fe^{3+}, Mg^{2+}-bimetal complex. Accordingly, Ca may be involved in the stabilization of the pigment.

Metal substitution experiments demonstrated that Ca in protocyanin can be substituted with Ba or Sr and also that Mg can be substituted with Mn, Co, Ni, Zn or Cd. Protocyanin is a high molecular-weight and complex pigment (Saito et al., 1961; Kondo et al., 1994) and in addition, application of NMR techniques to this pigment was not practical, as the compound contains Fe as an essential metal (Kondo et al., 1998). Thus, X-ray crystallography would be the most useful for elucidation of the total structure of protocyanin. In this study, we succeeded in the isolation of protocyanin in the form of crystals for the first time, and further investigation is now in progress using the crystals and the metal substituted complex pigments.

Table 2
The mole ratio of anthocyanin (**1**), flavone glycoside (**2**) and metal components contained in reconstructed and natural protocyanin

	1	**2**	Fe	Mg	Ca
Reconstructed protocyanin					
Sample 1	5.34	5.34	1	1.78	2.27
Sample 2	5.36	5.36	1	1.68	3.30
Sample 3	6.25	6.31	1	1.18	2.58
Mean	5.65	5.67	1	1.55	2.72
Natural protocyanin					
Sample 1	5.76	6.45	1	1.98	3.02
Sample 2	5.85	6.26	1	2.06	2.68
Mean	5.80	6.35	1	2.02	2.85

3. Experimental

3.1. General

UV–Vis and CD spectra were measured with a JASCO J-820 spectrometer, and Vis spectra with a Shimazu UV-160A spectrometer. A Hnu system 5000 energy dispersive X-ray analyzer was used for the measurements of metal elements. ICP spectroscopy was carried out using a Seiko SPS-1200A plasma spectrometer.

3.2. Isolation of pigments

Fresh blue flowers of *C. cyanus* were immersed in EtOH for dehydration, filtered and dried quickly. The dried material (1.8 kg) was finely powdered and the blue pigment was extracted twice with H_2O, 7.2 and 5.2 l, respectively. The blue extract was centrifuged at 10,000*g* for 20 min. After filtration, EtOH (2.5 vol) were added to the supernatant and a blue ppt. was collected by centrifugation at 3000*g* for 5 min (42.2 g). The crude pigment was dissolved in water (320 ml) and the deep blue solution was centrifuged at 5000*g* for 10 min to precipitate insoluble matter. An equal volume of EtOH was then added to the blue supernatant and the mixture was filtered. EtOH (450 ml) was added to the filtrate and the mixture was left overnight in a refrigerator. The blue pigment, protocyanin, precipitated was collected by filtration (9.1 g). This impure pigment was used for the experiments on the unknown factor, for further purification of protocyanin and also for preparation of **1** and **2**. Purification of protocyanin was carried out in a similar way as commelinin (Hayashi and Takeda, 1970) and protocyanin (Hayashi et al., 1961), that is, Sephadex LH20 CC (28 × 350 mm) eluted with H_2O and cellulose CC (28 × 150 mm) eluted with EtOH–H_2O (1:1), respectively, and then repeated precipitation of the pigment from aq. EtOH. Finally, purified protocyanin was separated as deep blue granules. The yield was ca. 28 mg from 1 g of the impure pigment.

For isolation of **1** and **2**, the impure pigment was dissolved in HCO_2H–MeCN–H_2O (8:20:72), to decompose the protocyanin, and left overnight in a refrigerator, whereby the color of the pigment changed to red. The red solution was subjected to preparation of **1** and **2** by HPLC on an ODS column (10 × 250 mm) using an isocratic elution with the following solvent system: HCO_2H–MeCN–H_2O (0.5:22:77.5). The HPLC step was repeated once more with the same solvent for **1** and **2**, respectively. Each pigment was passed through a LH 20 column (15 × 200 mm) with HCO_2H–MeCN–H_2O (0.5:20:79.5). The fraction containing the pigment was evaporated to dryness. After dissolving in a small amount of H_2O, each pigment was passed through a CM cellulose column (28 × 180 mm) slowly using H_2O as an eluant. Yield **1**: ca. 23 mg, **2**: ca. 25 mg from

Table 3
Substitution of Ca in the blue complex pigment with various kinds of metals

Metal used for substitution	Color of reaction mixtures	Blue pigment separable on Sephadex column	$\lambda_{vis\text{-}max}$ (nm) and $A_{vis\text{-}max}$ of the blue pigment
Ca[a]	Blue	+	574[b] (1.19[c]), 675[b] (0.39[c])
Mn	Dark blue	+	571 (0.20)
Co	Dark blue	+	584 (0.25)
Ni	Dark blue	+	577 (0.26)
Zn	Blue	+	577 (0.35), 673 (0.08)
Cd	Blue	+	577 (0.30), 674 (0.10)
Sr	Blue	+	576 (1.44), 673 (0.40)
Ba	Blue	+	576 (1.52), 676 (0.43)
Cu	Dark blue[d]	–	
K	Greenish blue[d]	–	
Na	Greenish blue[d]	–	

[a] Used as a control.
[b] Vis absorption maximum (nm).
[c] Absorbance at Vis_{max}.
[d] Faded on the column.

Table 4
Substitution of Mg in the blue complex pigment with various kinds of metals

Metal used for substitution	Color of reaction mixtures	Blue pigment separable on Sephadex column	$\lambda_{vis\text{-}max}$ (nm) and $A_{vis\text{-}max}$ of the blue pigment
Mg[a]	Blue	+	574[b] (1.23[c]), 675[b] (0.35[c])
Mn	Blue	+	577 (1.26), 676 (0.31)
Co	Blue	+	583 (1.87), 670[d] (0.41)
Ni	Purplish blue	+	580 (1.77), 672 (0.34)
Zn	Blue	+	576 (1.87), 673 (0.38)
Cd	Blue	+	579 (0.25), 673 (0.10)
Sr	Blue	+	581 (0.47)
Ba	Blue	+	578 (0.62)
Cu	Dark blue	–	
None[e]	Purplish blue	–	

[a] Used as a control.
[b] Vis absorption maximum (nm).
[c] Absorbance at Vis_{max}.
[d] Shoulder.
[e] Mixtures containing only Fe and Ca as metals.

200 mg of the blue pigment. **1** and **2** were also isolated from the dried petals. The pigment was extracted with HCO_2H–MeCN–H_2O (8:15:77). Filtered extracts were evaporated to a small volume and applied to purification by HPLC as described above. Yield **1**: ca. 17 mg, **2**: ca. 23 mg from 10 g of the dried petals.

3.3. Reconstruction of protocyanin

Experiments on the unknown factor and reconstruction of protocyanin were carried out principally as described previously (Takeda et al., 1984). Assay of the unknown factor was carried out as follows. To the mixture of **1** (0.35 μmol), **2** (0.35 μmol), $Fe(SO_4)$ (0.035 μmol) and $Mg(OAc)_2$ (0.70 μmol), 100 μl of each 4.5 ml fraction was added. Each mixture was evaporated to dryness and the residue was re-dissolved in a small volume of H_2O. Evaporation of the solution and re-solution of the residue were repeated five times. The blue aq. solution was then passed through a Sephadex LH 20 column (5 × 50 mm) using H_2O as an eluant. A blue fraction of the complex pigment that moved most rapidly and was easily separable from other materials was collected. The volume was adjusted to 1 ml with H_2O and the absorption spectrum in the visible region was measured. Reconstruction tests of protocyanin and metal substitution tests were carried out in the same way using purified **1** and **2**.

Isolation of reconstructed protocyanin in quantity was carried out in approximately the same way as for commelinin (Takeda and Hayashi, 1977), using **1** and **2** (30 μmol each), and metal ions, Fe, Mg and Ca

(0.1:2:3 in mole ratio to **1**). After the reaction as described above, the blue pigment synthesized was twice purified by Sephadex column chromatography (15×220 mm) and then an aq. solution of the blue pigment was added with EtOH (9 vol), whereby the blue pigment was precipitated. Precipitation of the pigments from an aq. ethanol solution was repeated several times and finally the reconstructed protocyanin was separated as crystals from aq. ethanol. The yield was ca. 8 mg.

3.4. Quantitative determination

The contents of **1** and **2** in the complex pigments were measured by the spectrophotometric method as described previously (Hayashi and Takeda, 1970), using the absorbances at 522 nm for **1** and 320 nm for **2**, respectively. The content of **2** was also determined by HPLC analysis (ODS column, 4.6×250 mm, 20% MeCN) after the treatment of protocyanin with HCO_2H–MeCN–H_2O (8:20:72) as reported previously (Takeda et al., 1994).

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