

Brilliant Skyblue Pigment Formation from Gardenia Fruits

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It is well known that blue pigment is formed by the reaction of amino acids with genipin, the hydrolyzate of geniposide from gardenia fruits. We studied the effect of the amino acid on blue pigment formation and found a linear relationship between the molecular weight of the neutral amino acid and the $\lambda_{\max}$ of the blue pigment formed. Thin layer chromatographic analysis revealed brilliant skyblue components of the blue pigments formed from glycine, alanine, leucine, phenylalanine and tyrosine. Furthermore, a brilliant skyblue color was obtained by a reverse phase column chromatography (HP-20) of blue pigments formed from glycine, leucine and phenylalanine. The $\lambda_{\max}$ of these purified pigments lay above 600 nm, and the peaks were sharper than those of crude pigments. After standing for two weeks at 40°C in 40% ethanol solution, the brilliant skyblue pigment formed from genipin and glycine remained stable, losing none of its initial absorbance.

The blue pigment produced from gardenia fruits (*Gardenia jasminoides*) is a natural pigment.¹⁾ Although it is very stable, its color is a dark blue.²⁾ A brilliant skyblue pigment of natural origin has not yet been obtained. The blue pigment is known to be formed from the geniposide contained in gardenia fruits, which is hydrolyzed by β -glucosidase to genipin, which in turn reacts with an amino acid to form the blue pigment.^{3,4)}

This blue pigment contains many blue components.⁵⁾ We studied the effect of the amino acid on the kind of blue pigment formed and found that the blue pigments formed by the reaction of genipin with neutral amino acids contained brilliant skyblue components, which could be purified by HP-20 chromatography.

Materials and Methods

Materials β -Glucosidase from almonds was purchased from Sigma chemical Co., Ltd. Gardenia fruits were purchased from Uchida Wakanyaku Co., Ltd. (Tokyo). Blue pigment from gardenia, phyco-

cyanin from blue green alga⁶⁾ and brilliant blue FCF (synthetic pigment) were obtained commercially.

Isolation of geniposide and genipin Geniposide was prepared by extraction of gardenia fruits with water, elution with 100% methanol from a charcoal column and elution with 85% chloroform in methanol from a silica gel.^{7,8)} Genipin was purified by hydrolyzing geniposide with β -glucosidase, extracting the hydrolyzate with ether, and crystalizing from methanol.

Analysis of geniposide and genipin The concentrations of geniposide and genipin were measured by high performance liquid chromatography (HPLC) on a YMC-ODS A-312 column [YMC Co., Ltd. (Kyoto)] with UV detector (240 nm). Elution was effected with 43% methanol at a flow rate of 1 ml/min. The retention times of genipin and geniposide were 8.3 and 5.8 min respectively.

Analysis of glycine The concentration of glycine was measured with a Hitachi amino acid analyzer 835-50.

Reaction of genipin with amino acids Genipin (1 or 10 mM) and the amino acid (1 or 10 mM) were mixed in MacIlvain's buffer (pH 7.0) and shaken for 4 h at 80°C.

Thin layer chromatographic analysis of blue pigment The sample was spotted on a silica-gel 60f 254 plate (10×10 cm) and developed with *n*-

butanol-acetic acid-water (4 : 1 : 2 by volume).

HP-20 chromatography of blue pigment The sample in aqueous solution was applied to an HP-20 column (Mitsubishi Kasei Co., Ltd., Tokyo). The sample absorbed on HP-20 was eluted with water, 20% methanol, 40% methanol and 100% methanol successively.

Evaluation of color The pigment was diluted to give an optical density (OD) of 1 at the maximum absorbance ($\lambda_{\max}$). The absorbance curve was measured from 400 to 700 nm with a Simadzu spectrophotometer UV-240.

Evaluation of pH stability The pH stability of each pigment was determined in MacIlvain's buffer (pH 3, 4, 5, 6, 7, 8) and MacIlvain's buffer containing 40% ethanol (pH 3, 4, 5, 6, 7, 8). The ratio of remaining absorbance at $\lambda_{\max}$ was determined after standing for two weeks at 40°C in the dark.

Results and Discussion

Color evaluation of blue pigment

The absorbance curves of blue pigment from gardenia fruits, phycocyanin and brilliant blue FCF are shown in Fig. 1. Visually, brilliant blue (FCF synthetic pigment) showed the most brilliant blue color, followed by phycocyanin, while the pigment from gardenia was dark blue. The absorbance curves of these three blue pigments showed that the $\lambda_{\max}$ and peak width at half height were respectively 590 nm and 116 nm for the blue pigment from gardenia, 615 nm and 80 nm for phycocyanin, and 625 nm and 45 nm for brilliant blue FCF. These data indicate that a high $\lambda_{\max}$ at 625 nm and narrow peak

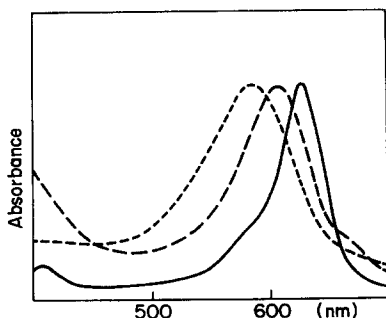


Fig. 1. Absorbance curves of blue pigments.
—, brilliant blue FCF; ---, phycocyanin;
- · - ·, blue pigment from gardenia.
Each pigment was dissolved in 40% ethanol.

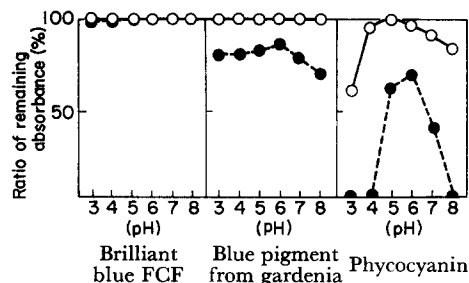


Fig. 2. Stability of blue pigments under various pH conditions. (Aqueous solution)
○, 0 time; ●, After 2 weeks at 40°C in the dark.
The pH was adjusted with MacIlvain's buffer.
The ratio of remaining absorbance was determined at $\lambda_{\max}$.

width at half height yield a brilliant skyblue color. We decided that blue pigments should be evaluated by $\lambda_{\max}$ and peak width at half height.

Evaluation of pH stability of blue pigment

The ratio of remaining absorbance of brilliant blue FCF, phycocyanin and blue pigment from gardenia upon standing for two weeks at 40°C at different pHs is shown in Fig. 2 for aqueous solutions and in Fig. 3 for solutions in 40% ethanol. The absorbance of brilliant blue FCF did not change, being very stable. That of blue pigment from gardenia was retained to the extent of 65–80% after two weeks, being relatively stable. But that of phycocyanin

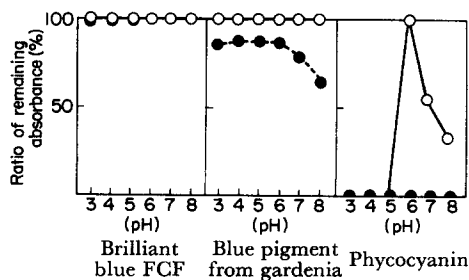


Fig. 3. Stability of blue pigments under various pH conditions. (40% ethanol solution)
○, 0 time; ●, After 2 weeks at 40°C in the dark.
The pH was adjusted with MacIlvain's buffer containing 40% ethanol. The ratio of remaining absorbance was determined at $\lambda_{\max}$.

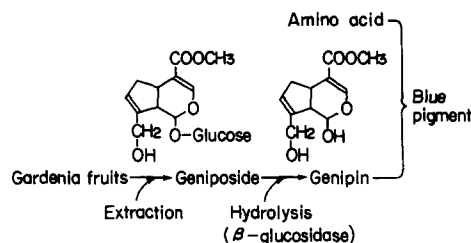


Fig. 4. Mechanism of blue pigment formation from gardenia fruits.

disappeared after standing for two weeks at pH 3–8 in ethanol or at pH 3, 4 or 8 in water.

Mechanism of blue pigment formation from gardenia fruits

Figure 4 shows the mechanism of blue pigment formation from gardenia fruits. Geniposide contained in gardenia fruits is hydrolyzed by β -glucosidase to its aglycone genipin, which reacts with primary amines to form a blue pigment. This blue pigment is a mixture of many components; and the structures of a few of the blue pigments formed from genipin with monomethylamine have been elucidated.⁵⁾

It is known that the type of amino acid affects the color of the blue pigment. We studied the relationship between the blue pigment obtained and the amino acid used and purified the blue pigments formed in order to obtain a brilliant skyblue pigment from gardenia fruits.

Blue pigment formed from various amino acids

Figure 5 shows the relation-

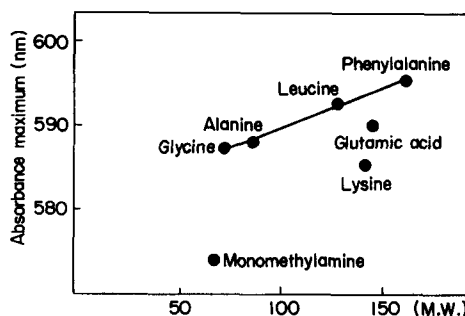


Fig. 5. Relationship between molecular weight of amino acid and $\lambda_{\max}$ of blue pigment.

Blue pigments were formed by shaking 10 mM solution of amino acids with genipin for 4 h at 80°C in MacIlvain's buffer (pH 7).

ship between $\lambda_{\max}$ of the blue pigment obtained and the molecular weight of the amino acid which reacted with genipin. A linear relationship was observed between the molecular weight of neutral amino acids and the $\lambda_{\max}$ of the blue pigments. No relationship was found between peak width at half height and the molecular weight of amino acid. The pigments formed with glycine, leucine and phenylalanine showed narrow peak widths at half height. That formed with phenylalanine gave the most brilliant skyblue color of the pigments studied.

Thin layer chromatography (TLC) analysis of blue pigment formed from genipin and various amino acids Using TLC, we attempted to detect a brilliant skyblue spot in the mixtures of blue pigments formed from genipin with various amino acids. Figure 6 shows that the blue pigments formed from genipin with glycine, alanine, leucine, phenylalanine and tyrosine possessed a high R_f brilliant skyblue spot on the thin layer chromatogram.

Purification of brilliant skyblue pigment using HP-20 TLC analysis of blue pigments showed that brilliant skyblue pigments are present in the blue pigments formed with glycine, alanine, leucine, phenylalanine and tyrosine. The blue pigments formed with glycine, leucine and phenylalanine were purified by HP-20 chromatography. Each fraction purified by HP-20

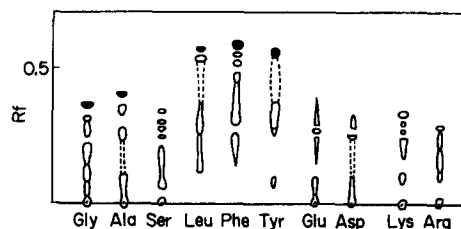


Fig. 6. Thin layer chromatographic analysis of blue pigment formed from various amino acids and genipin.

Samples were spotted on silica-gel 60f 254 plate (10×10 cm) and developed with *n*-butanol-acetic acid-water (4 : 1 : 2 by volume).

●, brilliant skyblue spot; ○, blue violet spot.

Table 1. Fractionation on HP-20 of blue pigment formed from glycine and genipin.

		Aqueous sol.		40% ethanol	
		$\lambda_{\max}$	P.W.	$\lambda_{\max}$	P.W.
Before separation		593 (nm)	95 (nm)	602 (nm)	80 (nm)
HP-20 separation	Water eluate	594	93	601	85
	40% MeOH eluate	594	90	602	78
	100% MeOH eluate	600	85	607	69

P.W.: Peak width at half height

Table 2. Fractionation on HP-20 of blue pigment formed from leucine and genipin.

		Aqueous sol.		40% ethanol	
		$\lambda_{\max}$	P.W.	$\lambda_{\max}$	P.W.
Before separation		598 (nm)	100 (nm)	603 (nm)	96 (nm)
HP-20 separation	Water eluate	597	106	600	98
	40% MeOH eluate	596	99	598	97
	100% MeOH eluate	599	96	606	90

P.W.: Peak width at half height

Table 3. Fractionation on HP-20 of blue pigment formed from phenylalanine and genipin.

		Aqueous sol.		40% ethanol	
		$\lambda_{\max}$	P.W.	$\lambda_{\max}$	P.W.
Before separation		600 (nm)	92 (nm)	603 (nm)	88 (nm)
HP-20 separation	Water eluate	598	100	600	95
	40% MeOH eluate	596	90	600	90
	100% MeOH eluate	600	90	607	84

P.W.: Peak width at half height

was dissolved in water and 40% ethanol. As shown in Tables 1, 2 and 3, the fraction eluted with 100% methanol from HP-20 contained a brilliant skyblue component. Significantly, $\lambda_{\max}$ and peak width at half height of the fraction eluted with 100% methanol showed the highest and narrowest values, [607 nm and 69 nm (glycine), 606 nm and 90 nm (leucine) and 607 nm and 84 nm (phenylalanine)] in 40% ethanol solution.

TLC analysis of the brilliant skyblue pigment purified by HP-20 The peak width at half height of brilliant skyblue pigment formed from genipin and glycine was narrower than those obtained with leucine and phenylalanine (Table 1). The brilliant skyblue pigment obtained by HP-20

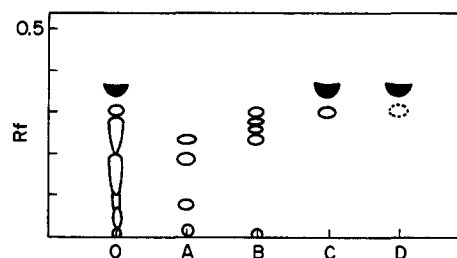


Fig. 7. Thin layer chromatographic analysis of fractions separated on HP-20.

Sample: 0, before separation (blue pigment formed from glycine with genipin); A, water elution; B, 20% methanol elution; C, 40% methanol elution; D, 100% methanol elution. Samples were spotted on silica-gel 60f 254 plate (10×10 cm) and developed with *n*-butanol-acetic acid-water (4 : 1 : 2 by volume).

●, brilliant skyblue spot; ○, blue violet spot.

purification of the blue pigment formed from genipin and glycine was analyzed by TLC. This analysis showed that the brilliant skyblue component was present in the fractions eluted with 40% and 100% methanol from the HP-20 column. This is why the 100% methanol fraction shows a brilliant skyblue color (Fig. 7).

Stoichiometry of the reaction of genipin with amino acids

Figure 8 shows the relationship between the molar fraction of genipin and the absorbance of the blue pigments formed by the reaction of genipin with amino acids. When the molar fraction of genipin is 0.5, a blue pigment having the maximum absorbance is produced. Figure 9 shows the molar concentration of remaining glycine in the reaction between genipin and glycine. When the molar concentration of glycine is higher than that of genipin, the molar concentration of remaining glycine is almost the same as the difference between the molar concentrations of genipin and glycine. When the molar concentration of glycine is lower than that of genipin, no remaining glycine is detected. These blue pigments are mixtures of many components as shown in Fig. 6. The results shown in Fig. 8 and Fig. 9 suggest that, overall, these mixtures of

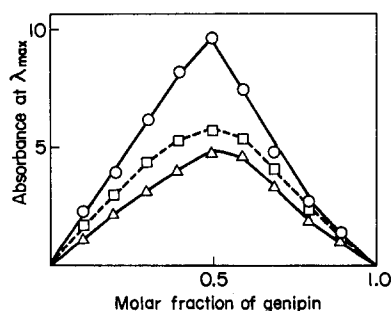


Fig. 8. Relationship between the molar fraction of genipin and the absorbance of the blue pigments formed by the reaction of genipin with amino acids.

○, glycine; △, leucine; □, phenylalanine.

Genipin and the amino acids were added to MacIlvain's buffer (pH 7) to give a total concentration of 1 mM, and these mixtures were shaken for 4 h at 80°C.

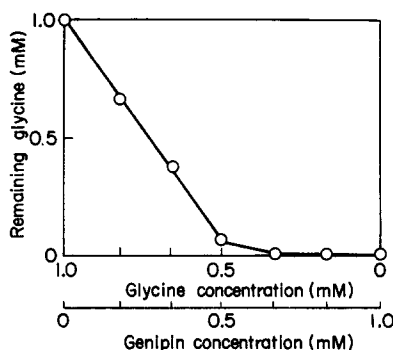


Fig. 9. Molar concentration of remaining glycine in the reaction of genipin with glycine.

Genipin and glycine were added to MacIlvain's buffer (pH 7) to give a total concentration of 1 mM, and the mixture was shaken for 4 h at 80°C.

blue pigments contain equimolar amounts of genipin and amino acid.

The pH stability of brilliant skyblue pigment

Figure 10 shows the pH stability of the brilliant skyblue pigment formed from genipin and glycine. Although this pigment showed decreased absorbance after

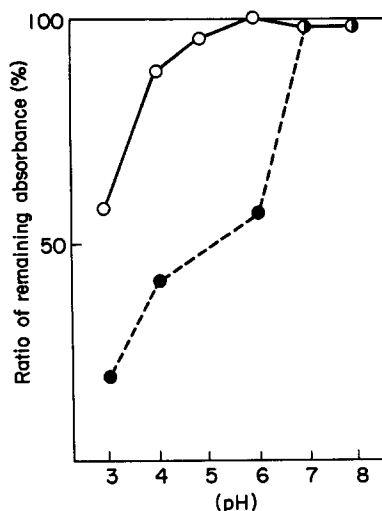


Fig. 10. Stability of brilliant skyblue pigment formed from genipin and glycine under various pH conditions. (Aqueous solution)

○, 0 time; ●, after 2 weeks at 40°C in the dark.

The pH was adjusted with MacIlvain's buffer. The ratio of remaining absorbance was determined at $\lambda_{\max}$.

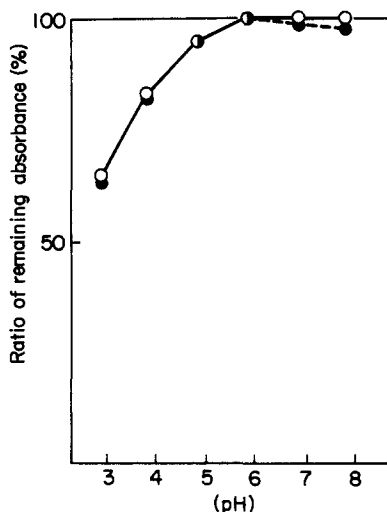


Fig. 11. Stability of brilliant skyblue pigment formed from genipin and glycine under various pH conditions. (40% ethanol solution)
○, 0 time; ●, after 2 weeks at 40°C in the dark. The pH was adjusted with MacIlvain's buffer containing 40% ethanol. The ratio of remaining absorbance was determined at $\lambda_{\max}$.

standing for two weeks at 40°C at low pH (3–6), the decrease was less than that of phycocyanin shown in Fig. 2. Figure 11 shows the pH stability of the same brilliant skyblue pigment after standing for two weeks

at 40°C in 40% ethanol solution. The absorbance of this pigment did not decrease, indicating that it was very stable compared with phycocyanin shown in Fig. 3.

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