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Separation and Characterization of Water Soluble Blue Pigments Formed from Geniposide of Gardenia Fruits

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Genipin, aglycone of geniposide isolated from fruits of *Gardenia jasminoides*, was transformed into blue pigments through reaction with glycine and methylamine. The blue pigments formed from glycine-reacted genipin were passed through Bio-Gel P-2 resin yielding fractions GG1 and GG2, and those from methylamine-reacted genipin were separated into fractions GM1-GM4. The first eluted higher molecular-weight fractions, GG1 and GM1, had higher tinctorial strength than the later eluted lower molecular-weight fractions, GG2 and GM2-GM4, respectively. ^{1}H-NMR spectra of GG1 and GM1 showed very broad peaks indicating that structures of the pigments were highly polymeric. ^{1}H-NMR spectra of GG2, GM3, and GM4 showed several sharp peaks at aliphatic and aromatic regions with accompanying broad peaks, although the spectrum of GM2 was rather simple. Determination of the structural and physical nature of the isolated pigments is in progress.

Key words: Gardenia jasminoides, *genipin, glycine, methylamine, gardenia blue pigments.*

Fruits of *Gardenia jasminoides*, which have been used traditionally as yellow dyes[1] for foods and fabrics in East Asia, are composed of crocin and related compounds.[1,2] Furthermore, they contain several colorless iridoid compounds such as geniposide, gardenoside, shanzhiside, gardoside, and methyldeacetylasperuloside,[1,2] which can be transformed into blue colorants in large quantities through simple modifications.[1] Geniposide **1** (Fig. 1) is the major component of iridoid glycosides of gardenia fruits. Hydrolysis of geniposide **1** by β-glucosidase yields genipin **2** (Fig. 1).[3] The reaction of genipin **2** with primary amine such as amino acids, taurine, and even proteins produces water-soluble blue pigments.[4] This simple process for the production of edible blue pigments from genipin and primary amines is currently used by the food industry. However, in spite of their wide use, structures of the blue pigments have not yet been clarified, although they are reported to be intractable mixtures of high molecular polymers.[5]

In this research we isolated geniposide **1** from gardenia fruits and hydrolyzed the compound with β-glucosidase. The resulting colorless genipin **2** was transformed into the blue pigments through the reaction with simplest amines, glycine, and methylamine to obtain clues for the structural characterization of the blue pigments. The transformed blue pigments were passed through Bio-Gel P-2 column, and the separated fractions were characterized by UV-vis and NMR spectrometries.

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Materials and Methods

Materials. Dried fruits of *G. jasminoides* were obtained from Kyungnam province and stored in the refrigerator. Silica gel for column chromatography (Kiesel gel 230-400 mesh) and TLC plate (Kiesel gel 60 F254) were purchased from Merck (Darmstadt, Germany). Bio-Gel P-2 (fractional range: 200-2,600) and Bio-Gel P-6 (fractional range: 1,000-6,000) resins were purchased from Pharmacia Fine Chemicals (Hercules, CA). β-Glucosidase and glycine were purchased from Sigma Chemical Co (Steinheim, Germany). Other chemicals including methylamine, charcoal, chloroform, and methanol were obtained from Aldrich (Milwaukee, USA) or Samchun Chemicals (Kyunggi, Korea).

Spectral Analysis. UV-vis spectra were recorded on a Milton Roy Spectronics 3000 spectrophotometer. ^{1}H-NMR and ^{13}C-NMR spectra were measured on a 400 MHz FT NMR (JEOL) at 400 and 100 MHz, respectively.

Isolation of Geniposide 1 and Genipin 2. Geniposide of *G. jasminoides* was isolated using the methods of Endo and Taguchi[3] and Lee *et al.*[6] with minor modifications. Geniposide in acetate buffer (pH 5.0, 37°C) was treated with β-glucosidase for 5 h to yield genipin, aglycone of geniposide. Detailed procedure for the preparation of geniposide and genipin and physical properties including UV-vis, and ^{1}H- and ^{13}C-NMR data were reported previously.[7]

Transformation and Separation of Gardenia Blue Pigments. Genipin (10 mg) in 2 m*l* of 100 mM phosphate buffer (pH 7.0, 70°C) was treated separately with 0.44 mmol each of glycine and methylamine for 5 h. Each transformed blue pigments from glycine and methylamine was passed

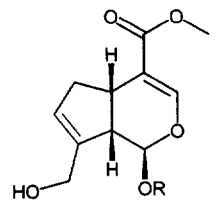

Fig. 1. Chemical structures of geniposide 1 and genipin 2.

through Bio-Gel P-2 resin (fractional range: 200-2,600) eluted with water. Two and four fractions of blue pigments formed from genipin with glycine and methylamine, respectively, were collected and measured using UV-vis and NMR spectroscopy to clarify their structures.

Results and Discussion

Geniposide **1** obtained from gardenia fruits was hydrolyzed with β-glucosidase, and the resulting genipin **2** was transformed into blue pigments through reactions with glycine and methylamine. UV-vis spectra during the transformation of the blue pigments from glycine-reacted genipin at a 1-min scanning interval showed that λ_{max} of genipin at 240 nm decreased,

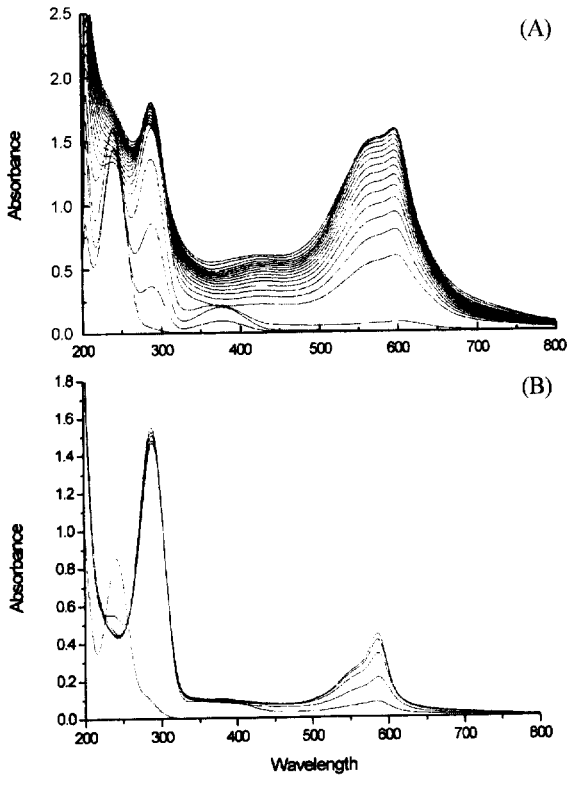

Fig. 2. (A) UV-vis spectra for the formation of blue pigments from glycine-reacted genipin in 100 mM phosphate buffer (pH 7.0, 70°C, scanning interval: 5 min). (B) UV-vis spectra for the formation of blue pigments from methylamine-reacted genipin in 100 mM phosphate buffer (pH 7.0, 70°C, scanning interval: 5 min).

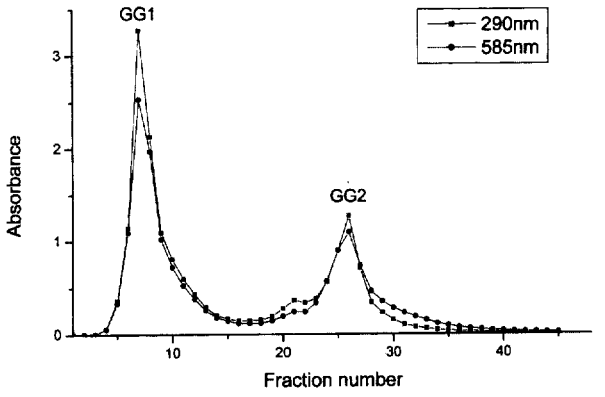

Fig. 3. Elution profile of Bio-Gel P-2 resin chromatography for the separation of blue pigments transformed from glycine-reacted genipin in 100 mM phosphate buffer (pH 7.0) at 70°C for 5 h: GG1 (fraction Nos. 6-10), GG2 (fraction Nos. 24-28).

while absorption of an intermediate peak at about 290 nm started to appear, and finally absorption at about 570-600 nm of blue pigment polymers produced (Fig. 2A).[6]

The transformed blue pigments from glycine-reacted genipin were passed through Bio-Gel P-2 resin (fractional range: 200-2,600) chromatography column. The column was first washed with one column volume of water, then eluted with 100 m*l* of water. Forty five fractions (2 m*l*/tube) of the blue pigments were collected and measured at 290 and 585 nm with a UV-vis spectrophotometer (Fig. 3). Two types of blue pigments, GG1 (fraction nos. 6-10) and GG2 (fraction nos. 24-28), were collected.

Figure 4 shows the UV-vis spectra of GG1 and GG2. Absorption maximum of GG1 (23 μg · ml^{-1} H_2O) at the blue-colored region was 584 nm with an absorbance of 0.639, while that of GG2 under the same condition was 589 nm with an absorbance of 0.049. These data indicated that the first eluted, higher molecular-weight fraction GG1 had higher tinctorial strength than the later eluted, lower molecular-weight fraction GG2.

^{1}H-NMR spectrum of GG1 fraction in D_2O showed very

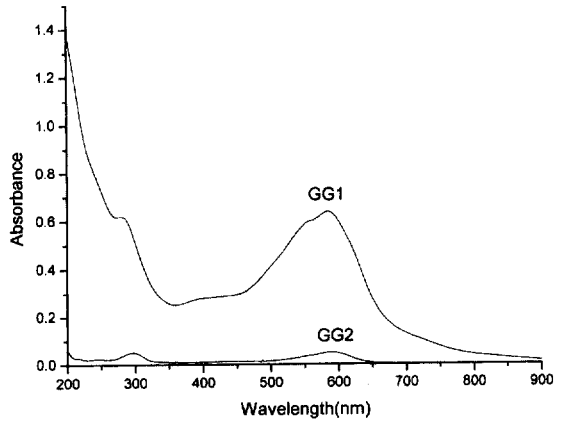

Fig. 4. UV-vis spectra of GG1 (23 μg · ml^{-1}) and GG2 (23 μg · ml^{-1}) in H_2O.

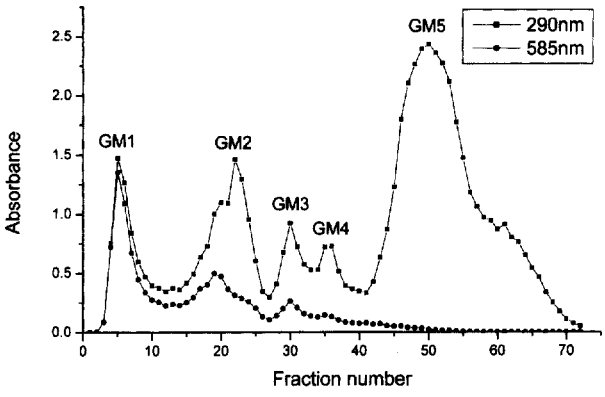

Fig. 5. Elution profile of Bio-Gel P-2 resin chromatography for the separation of blue pigments transformed from methylamine-reacted genipin in 100 mM phosphate buffer (pH 7.0) at 70°C for 5 h: GM1 (fraction Nos. 4-8), GM2 (fraction Nos. 17-25), GM3 (fraction Nos. 29-32), GM4 (fraction Nos. 34-37) and GM5 (fraction Nos. 44-57).

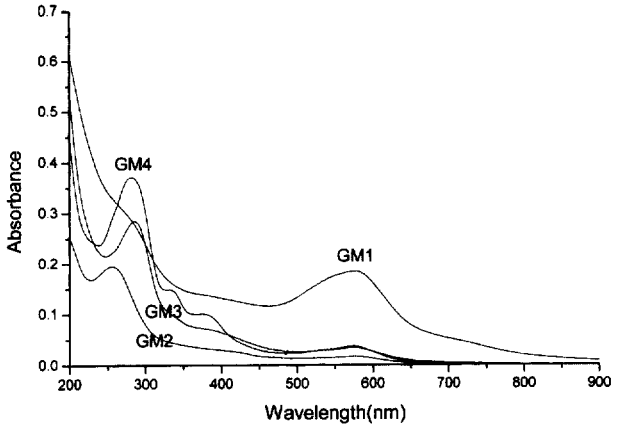

Fig. 6. UV-vis spectra of GM1, GM2, GM3 and GM4 in H_2O (13 μg · ml^{-1} each).

broad peaks at aliphatic and aromatic regions indicating the presence of polymeric compounds. No significant peaks were observed in ^{13}C-NMR spectrum. ^{1}H-NMR spectrum of GG2 fraction in D_2O showed several sharp peaks at aliphatic and aromatic regions with accompanying broad peaks which suggests the presence of polymeric compounds.

Fujikawa *et al.*[8] reported that blue dye from glycine-reacted genipin contained several blue components based on the thin layer chromatography results. However, the structures of the components were not mentioned.

Blue pigments from methylamine-reacted genipin (Fig. 2B) were passed through Bio-Gel P-2 resin chromatography eluted with water. Seventy five fractions (2 m*l*/tube) of the blue pigments were collected and measured at 290 and 585 nm with UV-vis spectrophotometer (Fig. 5). Four types of blue pigments, GM1 (fraction nos. 4-8), GM2 (fraction nos. 17-25), GM3 (fraction nos. 29-32), and GM4 (fraction nos. 34-37), from the methlyamine-reacted genipin were collected. GM5 (fraction nos. 44-57) was not a blue pigment.

Absorption maxima with the value of absorbance in parenthesis at blue-colored region of GM1-GM4 (each 1 3μg · ml^{-1} H_2O) were 574 (0.225), 581 (0.024), 582 (0.089), and 581 nm (0.086), respectively (Fig. 6). These data indicated that the first eluted, higher molecular-weight fraction GM1 had higher tinctorial strength than the later eluted, lower molecular-weight fractions GM2-GM4.

^{1}H-NMR spectrum of GM1 showed very broad peaks, which are indications of polymeric blue dye. ^{1}H-NMR spectra of GM3 and GM4 also showed polymeric broad peaks, although the spectrum of GM2 appeared rather simple. It was reported that precursors of the blue pigments produced by the reaction of genipin with methlylamine had a unique 2-methly-4-carbomethoxy-2-pyridine basic skeleton.5) The nature of the blue pigments is presently under detailed investigation.

For the molecular weight estimation, GM1 fraction was further passed through Bio-Gel P-6 resin (fractional range: 1,000-6,000). The broad elution profile of GM1 fraction through Bio-Gel P-6 resin (data not shown) indicated that this blue dye was a mixture of polymers with estimated molecular weights of several thousands.

Touyama *et al.*[5] reported that the blue pigments from methylamine-reacted genipin were an intractable mixture of high molecular-weight polymers with average molecular-weight of 8970 ± 600 on the basis of osmotic pressure measurement. Their data were slightly higher than our estimation; different procedures used for the blue pigments preparation might be one of the reasons. They prepared the blue pigments via two steps: A solution of genipin and methylamine in a mixture of McIlvaine buffer (pH 7.2, 20 m*l*), water (20 m*l*) and MeOH (20 m*l*) was stirred for 2.5 h at 50°C. On cooling, $CHCl_3$ extracts (brownish-red residue) in a mixture of water (20 m*l*) and MeOH (20 m*l*) heated for 6 h at 80°C. They concluded the blue pigments were a mixture of polymers consisting on average of 40-44 monomer units. Our data suggested that GM1 was a mixture of polymers consisting of 20-40 monomer units.

In summary, we prepared two types of blue pigments from genipin, obtained from geniposide of gardenia fruits, reacted with glycine and methylamine. The blue pigments formed from glycine-reacted genipin were chromatographed, yielding two types of blue pigments. Higher molecular-weight fraction GG1 (A_{584} = 0.639 in 23 μg · ml^{-1} H_2O) had higher tinctorial strength than the lower molecular weight fraction GG2 (A_{589} = 0.049 in 23 μg · ml^{-1} H_2O). The blue pigments formed from methylamine-reacted genipin were chromatographed yielding four fractions of blue pigments. The highest molecular-weight fraction GM1 (A_{574} = 0.225 in 13 μg · ml^{-1} H_2O) had the highest tinctorial strength than the lower molecular weight fraction GM2 (A_{581} = 0.024 in 13 μg · ml^{-1} H_2O), GM3 (A_{582} = 0.089 in 13 μg · ml^{-1} H_2O) and GM4 (A_{581} = 0.086 in 13 μg · ml^{-1} H_2O). Molecular-weight distribution of polymeric GM1 using Bio-Gel P-6 resin was found very broad, in the range of several thousands.

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