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Anthocyanin occurrence in the root peels, petioles and flowers of red radish (*Raphanus sativus* L.)

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Abstract

Three novel acylated pelargonidin 3-sophoroside-5-glucosides were isolated from the root peels, petioles and flowers of red radish, *Raphanus sativus* 'Cherry Mate', in addition to five known anthocyanins namely, pelargonidin 3-sophoroside-5-glucoside, pelargonidin 3-[2-(glucosyl)-6-(*trans*-*p*-coumaroyl)-glucoside]-5-glucoside, pelargonidin 3-[2-(glucosyl)-6-(*trans*-feruloyl)-glucoside]-5-glucoside, pelargonidin 3-[2-(glucosyl)-6-(*trans*-*p*-coumaroyl)-glucoside]-5-(6-malonylglucoside) and pelargonidin 3-[2-(glucosyl)-6-(*trans*-feruloyl)-glucoside]-5-(6-malonylglucoside). The structures of three new acylated anthocyanins were shown to be pelargonidin 3-*O*-[2-*O*-(β-D-glucopyranosyl)-6-*O*-(*trans*-caffeoyl)-β-D-glucopyranoside]-5-*O*-(6-*O*-malonyl-β-D-glucopyranoside), its demalonyl derivative, and pelargonidin 3-*O*-[2-*O*-(β-D-glucopyranosyl)-6-*O*-(*cis*-*p*-coumaroyl)-β-D-glucopyranoside]-5-*O*-(6-*O*-malonyl-β-D-glucopyranoside). These pigments were the main components present not only in the root but also in the petioles and flowers of red radish. *p*-Coumaroyl anthocyanins were the main pigments found in the root, petioles and flowers. Although the *trans*-*p*-coumaroyl form was abundant in all three plant organs, its *cis* form was present in very low amount within the root but in large amount in the flowers and petioles.

Keywords: *Raphanus sativus* L.; Cruciferae; Pelargonidin 3-sophoroside-5-glucoside; *trans*- and *cis*-*p*-coumaric acids; Caffeic; Ferulic and malonic acids

1. Introduction

Radish (*Raphanus sativus* L.) root anthocyanins have been characterized by several researchers [1–4], who determined the presence of pelargonidin 3-sophoroside-5-glucoside derivatives acylated with *p*-coumaric, caffeic and ferulic acids. Recently, 16 acylated anthocyanins from radish roots have also been fully characterized as 3-mono- or di-hydroxycinnamoyl (*p*-coumaric, caffeic and/or ferulic acid)-sophoroside-5-glucoside, 3-mono- or di-hydroxycinnamoyl (*p*-coumaroyl, feruloyl, or di-feruloyl)-sophoroside-5-malonylglucoside, 3-mono- or di-feruloyl-sophoroside-5-malonylglucosylglucoside and 3-di-feruloyl-sophoroside-5-glucosylglucoside of pelargonidin and 3-caffeoyl-feruloyl-sophoroside-5-glucoside of cyanidin [5–7] by spectroscopic analyses including mass spectrometry and NMR technique. However, the complete structural determination of an anthocyanin acylated with caffeic acid or *cis*-*p*-coumaric acid together with malonic acid from the radish has not been reported to date. Although the presence of anthocyanins in the root peels of red radish has been investigated, the occurrence of anthocyanins in the flowers and petioles has not been reported yet. In this paper, we wish to report the complete structural determination of three new acylated anthocyanins. During our investigation of the new anthocyanins, we recognized that the pigments previously discovered in the root were also present in the petioles and flowers.

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2. Experimental

2.1. Plant materials

Red radish (*R. sativus* L.) cultivars 'Cherry Mate' (Tohoku Co., Ltd., Utsunomiya, Japan) and 'Flamboyant Sabina' (Thompson & Morgan Ltd., England) were cultivated in a green house on the Experimental Farm of Minami-kyushu University from November 2006 to April 2007. The roots of 'Cherry Mate' and 'Flamboyant Sabina' were harvested in December 2006. The roots' peels were dried overnight at 40 °C and kept in a refrigerator at 4 °C. The petioles and flowers (acyanic flowers were excluded) of 'Cherry Mate' were harvested in January 2007 and April 2007, respectively. They were dried and kept along with the root peels until their use in the experiment.

2.2. General procedures

TLC was carried out on plastic coated cellulose sheets (Merck) using nine mobile phases: BAW (*n*-BuOH–HOAc–H_2O, 4:1:2, v/v/v), BuHCl (*n*-BuOH–2 N HCl, 1:1, v/v, upper layer), AHW (HOAc–HCl–H_2O, 15:3:82, v/v/v), 1% HCl and Forestal (HOAc–HCl–H_2O, 30:3:10, v/v/v) for anthocyanins, and BAW, APW (EtOAc–pyridine–H_2O, 15:7:5, v/v/v), EAA (EtOAc–HCOOH–H_2O, 5:2:1, v/v/v), BEW (*n*-BuOH–EtOH–H_2O, 4:1:2.2, v/v/v) and 15% HOAc (HOAc–H_2O, 15:85, v/v) for sugars and organic acid with UV light and aniline hydrogen phthalate spray reagent [8].

Analytical HPLC was performed on an LC 10A system (Shimadzu), using a Waters C18 (4.6 ∅ × 250 mm) column at 40 °C with a flow rate of 1 mL min^{-1} and monitoring at 530 nm. The eluant was applied as a linear gradient elution for 40 min from 20 to 85% solvent B (1.5% H_3PO_4, 20% HOAc, 25% MeCN in H_2O) in solvent A (1.5% H_3PO_4 in H_2O). Prep. HPLC was performed on a Waters C18 (19 ∅ × 150 mm) column at 40 °C with a flow rate of 4 mL min^{-1} and monitoring at 530 nm. The solvent used was as follows: a linear gradient elution for 15 min from 60 to 70% solvent B in solvent A. UV–vis spectra were recorded on MPS-2450 (Shimadzu) in 0.1% HCl–MeOH (from 200 to 700 nm). FAB mass spectra were measured with a JEOL SX 102 in the positive ion mode using the magic bullet (5:1 mixture of dithiothreitol and dithioerythritol) as a matrix. NMR spectra were measured at 500 MHz for ^1H spectra and at 125.78 MHz for ^{13}C spectra on JEOL LA 500 instrument in DMSO-d_6–CF_3COOD (9:1). Chemical shifts are reported relative to a TMS internal standard (δ), and coupling constants are in hertz.

2.3. HPLC analysis of anthocyanins

Dried root peels, petioles and cyanic flowers (*ca.* 5–10 mg) of 'Cherry Mate' and root peels from 'Flamboyant Sabina' were extracted with 1 mL MAW (MeOH–HOAc–H_2O, 4:1:5, v/v/v). Analysis of anthocyanins was performed on these crude extracts (20 μL each) by HPLC.

2.4. Extraction and purification of anthocyanins

Dried root peels (*ca.* 20 g) from *R. sativus* 'Cherry Mate' were immersed in 5% HOAc–H_2O (1L; HOAc–H_2O, 1:19, v/v) at room temperature for 5 h and extracted with the same solvent. The extract was adsorbed on a Diaion HP-20 (Mitsubishi Chemical's Ion Exchange Resins) column, and the column was washed with H_2O. The pigment was eluted from the column with 5% HOAc–MeOH (500 mL; MeOH–HOAc, 95:5, v/v). After concentration, the eluates were fractionated with paper chromatography (PC) using BAW. The crude fractionated pigment obtained was further purified by TLC (15% HOAc) and prep. HPLC. Each fraction was absorbed on a Diaion HP-20 column, and washed with H_2O to free from MeCN and H_3PO_4. The anthocyanins were eluted with 5% HOAc–MeOH from the column. Concentrated anthocyanin residues were dissolved in a small volume of 5% HOAc–EtOH, followed by the addition of excess Et_2O to give eight precipitated anthocyanins: pigments **1** (*ca.* 5 mg), **2** (*ca.* 3 mg), A (*ca.* 1 mg), B (*ca.* 1 mg), C (*ca.* 1 mg), D (*ca.* 10 mg), E (*ca.* 3 mg) and demalonyl pigment **1** (*ca.* 0.5 mg).

2.5. Chemical and spectroscopic analyses of purified anthocyanins

Characterization of these pigments (**1**, **2**, A–E and demalonyl pigment **1**) was carried out using the standard methods [8,9], and the data obtained are summarized in Table 1. Acid hydrolysis of pigments **1** (*ca.* 1 mg) and **2** (*ca.* 1 mg) was carried out with 2 N HCl (2 ml) at 100 °C for 1 h, to provide pelargonidin, glucose, caffeic acid and malonic acid from pigment **1**, and pelargonidin, glucose, *cis*-*p*-coumaric acid and malonic acid from pigment **2**. These compounds were confirmed by direct comparison with the authentic samples using TLC and/or HPLC. Moreover, the demalonylation of pigment **1** was carried out as the following process: pigment **1** (*ca.* 1 mg) was dissolved in 1 N HCl solution at room temperature for 5 days [10]. After disappearance of the starting material by HPLC (Fig. 1), demalonyl pigment **1** was isolated and purified from the hydrolysate by TLC (BAW and AHW). Alkaline hydrolysis of pigments **1** (*ca.* 1 mg) and **2** (*ca.* 1 mg) was carried out with 2 N NaOH solution (1 mL) under N_2 gas at ambient temperature for 15 min to give one deacylated anthocyanin, whose structure was identified to be pelargonidin 3-sophoroside-5-glucoside in comparison with the authentic sample, obtained from *Ipomoea purpurea* [11]. The structure confirmation of pigments A–E from 'Cherry Mate' was performed by the analysis of TLC, HPLC and spectrophotometric measurements with authentic anthocyanins purified from the root peels of 'Flamboyant Sabina' as the same procedure [5,12]. In order to obtain the *cis* isomer of pigment D, pigment D was dissolved in 5% HOAc–MeOH (0.1×10^{-5} M) and exposed to direct sunlight for 10 min [13] on December 8, 2006. After transformation of about 50% of the starting material was observed on TLC, the isomer was isolated and purified by TLC and HPLC to provide the pure *cis* isomer D (*ca.* 3 mg).