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Acylated cyanidin 3-sophoroside-5-glucosides from the purple flowers of *Raphanus sativus* L. var. *raphanistroides* Makino (Brassicaceae)

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ABSTRACT

Two novel acylated cyanidin 3-sophoroside-5-glucosides were isolated from the purple flowers of *Raphanus sativus* L. var. *raphanistroides* Makino (Family: Brassicaceae). Chemical and spectroscopic methods revealed that they were cyanidin 3-*O*-[2-*O*-(2-*O*-(*trans*-caffeoyl)-β-glucopyranosyl)-6-*O*-(*trans*-*p*-coumaroyl)-β-glucopyranoside]-5-*O*-[6-*O*-(malonyl)-β-glucopyranoside] and cyanidin 3-*O*-[2-*O*-(2-*O*-(*trans*-caffeoyl)-β-glucopyranosyl)-6-*O*-(*trans*-feruloyl)-β-glucopyranoside]-5-*O*-[6-*O*-(malonyl)-β-glucopyranoside], which are major floral anthocyanins. In addition, mono-hydroxycinnamoyl cyanidin 3-sophoroside-5-malonyl-glucosides, which have been identified as root peel anthocyanins of purple radishes, were identified as minor anthocyanins in the flowers of *R. sativus* var. *raphanistroides* using comparative high performance liquid chromatography with root peel anthocyanins.

1. Introduction

The genus *Raphanus* includes eight species of annual and perennial herbs that are native to Europe and Asia. In Japan, *R. sativus* L. var. *raphanistroides* Makino (Japanese: Hamadaikon, English: Japanese wild radish) is only found as a wild plant most commonly on sandy coasts, estuaries, brackish water lakesides, and riverbanks. Several research groups have identified radish anthocyanins and the presence of acylated 3-sophoroside-5-glucosides of cyanidin and pelargonidin have been reported for red and purple root peel of radish cultivars (Ishikura and Hayashi, 1962, 1963; Guisti et al., 1998a,b; Mori et al., 2006; Otsuki et al., 2002; Tamura et al., 2010; Tatsuzawa et al., 2008a, 2010a; Kato et al., 2013). Although the presence of acylated pelargonidin 3-sophoroside-5-glucosides in the red flowers of *R. sativus* has been reported previously (Tatsuzawa et al., 2008a), the occurrence of anthocyanins in its purple flowers has not been reported yet. In this paper, I report the complete structural determination of two new acylated cyanidin 3-sophoroside-5-glucosides, together with four known ones, from the purple flowers of *R. sativus* var. *raphanistroides*. In addition, the acylated cyanidin 3-sophoroside-5-glucosides of the purple flowers from the two cultivars 'Benikanmi' (type: Japanese Radish cultivar) and 'Amethyst' (type: European radish cultivar) are compared.

2. Results and discussion

Two major anthocyanin peaks (**1** and **2**) at 530 nm (Fig. 1), with many minor peaks including four known anthocyanins (A–D) which were identified as major anthocyanins in purple roots of radishes (Tatsuzawa et al., 2010a) (Fig. 2), were found in the 5% HOAc (acetic acid-water = 5:95, v/v) extracts (2L) from dried purple flowers of *R. sativus* var. *raphanistroides* (100 g), by high performance liquid chromatography (HPLC) analysis.

The major peaks (**1** and **2**) were extracted from the purple flowers with 5% HOAc, followed by isolation using Diaion HP-20 (Nippon Rensui Co., Tokyo, Japan) column chromatography (CC), paper chromatography (PC), and preparative HPLC (Tatsuzawa et al., 2008a,b). The chromatographic and spectroscopic properties of **1** and **2** are summarized in sections 4–3–1 and 4–3–2.

Acid hydrolysis of **1** and **2** resulted in cyanidin, glucose, caffeic acid, and malonic acid. Moreover, *p*-coumaric acid was detected in the hydrolysate of **1** and also ferulic acid was detected in the hydrolysate of **2**. These acid hydrolysates were identified by direct comparison of TLC and HPLC with the commercial standards (Wako Pure Chemical Industries Co. Ltd., Tokyo, Japan) (Tatsuzawa et al., 2010a).

Alkaline hydrolysis of **1** and **2** resulted in only one deacylanthocyanin, whose structure was identified to be cyanidin 3-sophoroside-5-glucoside by direct comparison of TLC and HPLC with authentic sample obtained from the purple root peel anthocyanin of *R. sativus* 'Benikanmi' by alkaline hydrolysis

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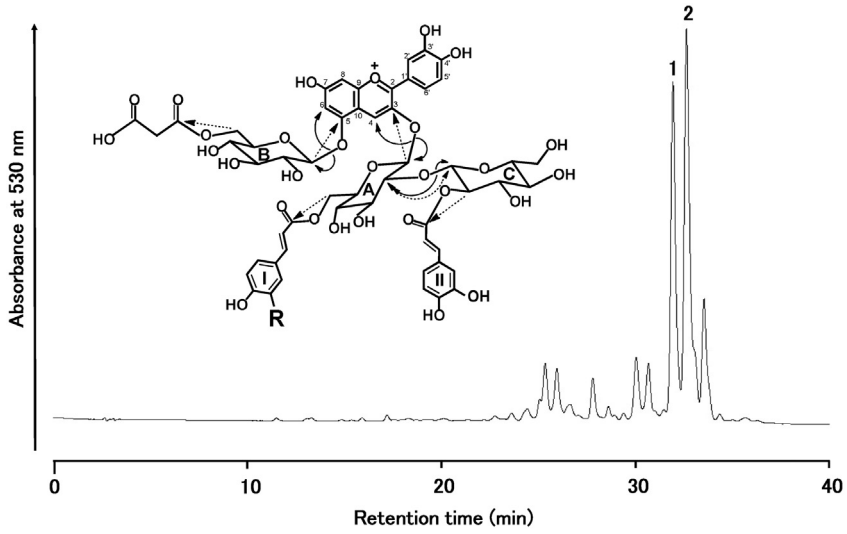

Fig. 1. HPLC profile (530 nm) and structure of acylated anthocyanins (**1** and **2**) isolated from the purple flowers of *Raphanus sativus* var. *raphanistroides*.
Observed NOEs of **1** are indicated by arrows.
Observed HMBCs of **1** are indicated dotted arrows.
1: pigment **1**, R = H {cyanidin 3-*O*-[2-*O*-(2-*O*-(*trans*-caffeoyl)-glucosyl)-6-O-(*trans*-*p*-coumaroyl)-glucoside]-5-*O*-[6-*O*-(malonyl)-glucoside]}
2: pigment **2**, R = OCH_3{cyanidin 3-*O*-[2-*O*-(2-*O*-(*trans*-caffeoyl)-glucosyl)-6-*O*-(*trans*-feruloyl)-glucoside]-5-*O*-[6-*O*-(malonyl)-glucoside]}

(Tatsuzawa et al., 2010a). Moreover, the alkaline hydrolysates, malonic and caffeic acid were detected in the hydrolysates of **1** and **2**, and also *p*-coumaric acid was detected in the hydrolysate of **1** and also ferulic acid was detected in the hydrolysate of **2**.

The structures of **1** and **2** were confirmed based on the analyses of their ^{1}H (400 MHz), ^{13}C (100 MHz) and 2D (COSY, NOESY, ^{1}H–^{13}C HMQC and ^{1}H–^{13}C HMBC) NMR (JNM AL-400, JEOL Ltd., Tokyo, Japan) spectra in DMSO-d_6-CF_3COOD (9:1), as well as their high resolution fast atom bombardment mass spectra (HR–FABMS) (LMS-700, JEOL Ltd.).

2.1. Pigment 1

The molecular ion $[M]^+$ of **1** was observed at *m*/*z* 1167 ($C_{54}H_{55}O_{29}$) indicating that **1** was composed of cyanidin with three molecules of glucose, one molecule each of *p*-coumaric acid, caffeic acid, and malonic acid. The elemental components were confirmed by measuring its HR-FABMS, and the mass data are summarized in Section 4.3.1. The structure was elucidated based on the analysis of the NMR spectra (Table 1).

The chemical shifts of 6 aromatic protons of cyanidin moiety with their coupling constants were assigned as shown in Table 1. Two sets of two pairs of doublet resonances, assigned to the four olefinic proton signals of the *p*-coumaric acid and caffeic acid moieties, indicated *trans* configuration for the acids based on their coupling constants (J = 15.8 Hz each)(Table 1). The chemical shifts of the sugar moieties were observed in the region of δ 5.68–3.08, where the three anomeric protons exhibited at δ 5.68 (d, J = 7.3 Hz, Glc A), δ 5.20 (d, J = 7.3 Hz, Glc B), and δ 5.21 (d, J = 7.3 Hz, Glc C). Based on the observed coupling constants (Table 1), glucoses were assumed to be in the β-pyranose form.

Based on results of analyses of their COSY spectra, characteristic five protons shifted to the lower magnetic fields were assigned to a methine proton of Glc C (δ 4.67) and methylene protons of Glc A (δ 4.27 and 4.41) and Glc B (δ 4.08 and 4.42). These results revealed that the OH-2 of Glc C and OH-6 of Glc A and B were acylated by three acid residues (caffeic acid, *p*-coumaric acid, or malonic acid). A signal appeared at δ 4.17 (t, J = 8.2 Hz, H-2 of Glc A) was easily correlated to the proton H-1 of Glc A. Thus, this proton was assigned to the H-2 of Glc A. The signal of the anomeric proton of Glc C correlated to the signal of the H-2 proton of Glc A in the NOESY spectrum. These results suggested that Glc C attached to OH-2 of Glc A through a glucosidic bond, and formed a sophorose unit. Moreover, NOESY and HMBC spectra were used to determine the sites of attachment of the acids, sugars, and cyanidin aglycone (Fig. 1). The signals of H-1s of Glc A and B were correlated to the signal of C-3 (δ 144.6) and C-5 (δ 155.0) carbons of cyanidin in their HMBC spectra, and also to the signals of the H-4 and H-6 protons of cyanidin in NOESY spectra. The signals of H-6s of Glc A and B, and H-2 of Glc C were correlated to the signals of COOH carbons of *p*-coumaric acid and malonic acid, and caffeic acid, respectively, in the HMBC spectra.

Consequently, the structure of **1** was determined to be cyanidin 3-*O*-[2-*O*-(2-*O*-(*trans*-caffeoyl)-β-glucopyranosyl)-6-*O*-(*trans*-*p*-coumaroyl)-β-glucopyranoside]-5-*O*-[6-*O*-(malonyl)-β-glucopyranoside] (Fig. 1), which is a new anthocyanin in plants (Andersen and Jordheim, 2006; Harborne and Baxter, 1999; Veitch and Grayer, 2008, 2011).

2.2. Pigment **2**

The molecular ion $[M]^+$ of **2** was observed at *m*/*z* 1197 ($C_{55}H_{57}O_{30}$) indicating that **2** was composed of cyanidin with three molecules of glucose, one molecule each of ferulic acid, caffeic acid, and malonic acid. The elemental components were confirmed by measuring its HR-FABMS, and the mass data are summarized in Section 4.3.2. The structure was elucidated based on the analysis of the NMR spectra (Table 1).

The ^{1}H NMR spectra of **2** showed the presence of one molecule each of cyanidin, caffeic acid, ferulic acid, and malonic acid, and three molecules of glucose. By the analysis of its ^{1}H and ^{13}C NMR spectra, it was revealed that chemical shifts of **2** were almost in agreement with those of **1**, with **2** containing a ferulic acid moiety instead of a *p*-coumaric acid moiety (Table 1). Consequently, **2** was determined to be cyanidin 3-*O*-[2-*O*-(2-*O*-(*trans*-caffeoyl)-β-glucopyranosyl)-6-*O*-(*trans*-feruloyl)-β-glucopyranoside]-5-*O*-[6-*O*-