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Nasunin from Eggplant Consists of Cis−Trans Isomers of Delphinidin 3-[4-(*p*-Coumaroyl)-L-rhamnosyl (1→6)glucopyranoside]-5-glucopyranoside

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Two major anthocyanins were isolated from the acidified methanolic extract of eggplant (*Solanum melongena* L.) by column chromatography and preparative high-performance liquid chromatography. These anthocyanins were interconvertible under room light illumination condition. By means of tandem time-of-flight mass spectrometry and nuclear magnetic resonance spectroscopy, their structures were identified and elucidated as delphinidin 3-[4-(*cis*-*p*-coumaroyl)-L-rhamnosyl(1→6)glucopyranoside]-5-glucopyranoside (compound **1**) and delphinidin 3-[4-(*trans*-*p*-coumaroyl)-L-rhamnosyl-(1→6)glucopyranoside]-5-glucopyranoside (compound **2**), respectively. The results indicated that nasunin comprised cis and trans isomers of the *p*-coumaric acid moiety in its structure.

KEYWORDS: Anthocyanin; eggplant; nasunin; *p-cis*-coumaric acid; *p-trans*-coumaric acid; tandem MS; NMR; isomerization

INTRODUCTION

Anthocyanins are reddish pigments widely distributed in a variety of plant materials such as colored fruits (*1*−*4*) and vegetables (*5*−*8*) and have attracted attention on the basis of their health-promoting benefit. Physiological functions of anthocyanins such as antioxidant activity (*9*−*13*), improvement of vision (*14*, *15*), and anticancer effect (*16*−*18*) have been reported. Studies on anthocyanin absorption and metabolism have also been carried out in both experimental animals and human subjects to determine their health benefits (*19*−*24*). As a result, anthocyanins were found to be absorbed in their intact glycoside forms into blood plasma, suggesting that the original structure contributed to their physiological function in vivo and, thus, anthocyanins are major functional food compounds, like other flavonoids such as quercetin and catechins. Normally, anthocyanidins are present as glycosides and additional acyl substituents. Various types of acylated anthocyanins were found in flowers (*25*−*27*) and certain types of edible plants (*5*, *6*, *8*). They are relatively stable compared to nonacylated anthocyanins, which quickly degrade in physiological conditions (*28*, *29*). One of the reasons for their stability has been explained by intramolecular stacking (*29*). Thus, the health benefit of acylated anthocyanins has attracted much attention. Terahara et al. (*6*) isolated acylated anthocyanins from purple sweet potato and reported their unique function, such as α-glucosidase inhibitory activity, that might be beneficial for the prevention of diabetes (*30*, *31*). Recently, in vitro antioxidant activity of anthocyanins from purple sweet potato was also reported (*32*).

Eggplant is one of the most widespread and common vegetables in the world. The color of eggplant is due to anthocyanins. The major anthocyanin in eggplant was primarily purified (*33*) and identified (*34*, *35*) as nasunin (delphinidin 3-[4-(*p*-coumaroyl)-L-rhamnosyl-(1→6)glucopyranoside]-5-glucopyranoside). The radical scavenging activity of nasunin was also well studied using an electron spin trapping method by Noda et al. (*36*, *37*).

However, we found, in the present study, that there exist two major anthocyanins in eggplant peel. It is interesting to know how this finding relates to nasunin reported previously (*34*, *35*). Because nasunin has a *p*-coumaroyl moiety in its structure, cis and trans configrations will occur. Therefore, we here carried out further structural assignment of two anthocyanins in eggplant. Such information will be valuable for the studies on absorption and metabolism of eggplant anthocyanins.

MATERIALS AND METHODS

Chemicals. All reagents including trifluoroacetic acid (TFA) were of HPLC or special grade and purchased from Wako Pure Chemical Ind., Osaka, Japan, and were used without further purification.

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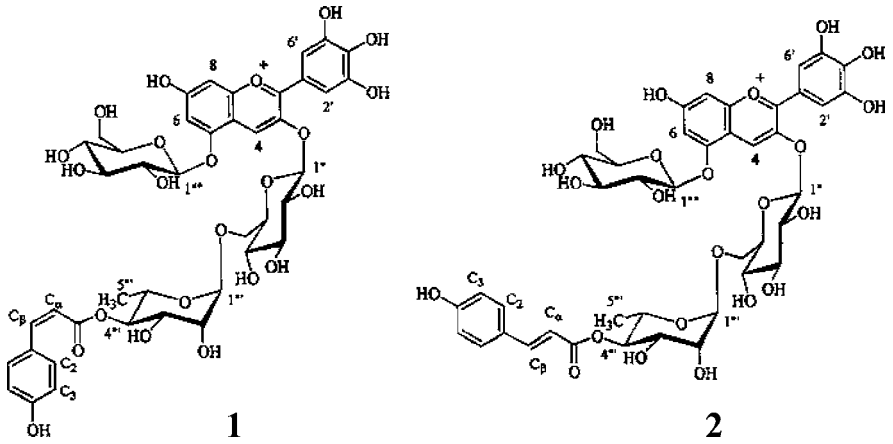

Figure 1. Structures of eggplant anthocyanins: **1**, delphinidin 3-[4-(*cis-p*-coumaroyl)-L-rhamnosyl(1→6)glucopyranoside]-5-glucopyranoside; **2**, delphinidin 3-[4-(*trans-p*-coumaroyl)-L-rhamnosyl-(1→6)glucopyranoside]-5-glucopyranoside.

Extraction and Purification of Eggplant Anthocyanins. Eggplant (10 kg) was obtained from a local market in Niigata prefecture, Japan, in August 2002. The peel of the eggplant was immersed in 10 L of methanol (MeOH) containing 3% TFA, and the resulting mixture was kept overnight in a refrigerator for anthocyanin extraction and evaporated to dryness in vacuo at 40 °C. An aliquot of this residue was redissolved in 0.5% aqueous TFA and analyzed by HPLC as described below. The residue was dissolved in distilled water and applied to a 100 cm × 7 cm HP-20 column (Mitsubishi, Tokyo, Japan). The column was well washed with distilled water to remove water-soluble compounds, and then the anthocyanin-containing fraction was eluted with 50% MeOH. The MeOH was removed from the collected fraction in vacuo at 40 °C. The anthocyanin solution was applied onto a 50 cm × 3.5 cm open column packed with MCI gel (Mitsubishi) and separated by H_2O as elution solution with increasing MeOH concentration (from 0:1 to 1:0). Aliquots of the anthocyanin fraction obtained were further purified by semipreparative HPLC equipped with a 250 mm × 20 mm i.d. Develosil ODS HG-5 column (Nomura Chemical, Seto, Japan) using 33% MeOH containing 0.5% TFA as elution solvent at a flow rate of 7 mL/min. The peak fraction was evaporated to dryness in vacuo. The purity of collected anthocyanins was checked with analytical HPLC described below.

Analytical HPLC. The analysis of anthocyanins contained in eggplant peel was carried out using an L-7000 HPLC system (Hitachi, Tokyo, Japan) equipped with a 150 mm × 4.6 mm i.d. Develosil ODS HG-5 column (Nomura Chemical). HPLC was run by linear gradient elution mode using 0.5% TFA aqueous (solvent A) and MeOH containing 0.5% TFA (solvent B). The gradient condition was as follows: 75% A/25% B (v/v) for 40 min and 75% A/25% B (v/v) to 30% A/70% B (v/v) for 15 min and then held at 30% A/70% B (v/v) for a further 10 min, at a flow rate of 2.0 mL/min. The elution profile was monitored at 520 nm.

Identification of Eggplant Anthocyanins. Eggplant anthocyanins were identified by tandem TOF MS and NMR spectrometry. Each sample was dissolved in MeOH and subjected to mass spectrometry using an Q-Tof Ultima (Micromass, Manchester, U.K.). The conditions for TOF MS-MS were as follows: A syringe pump (KD Scientific Inc., Holliston MA) was used to provide a constant infusion (300 μL/h) of the sample into the MS ion source. MS parameters used were as follows: 3.2 kV for capillary; 9.1 kV for reflection. Argon gas was used for collision at a pressure of 11 psi, and the applied voltage was 28 V. ^{1}H and ^{13}C NMR spectra were measured by a JEOL-ECA-500 NMR spectrometer (JEOL, Tokyo, Japan) at a magnetic field strength of 500 or 125 MHz, respectively, in TFA-d_1/CD_3OD-d_4 (1:9) using tetramethylsilane as an internal reference.

Conditions for Isomerization Reaction. After the NMR measurement, a sample solution containing compound **1** (**Figure 1**) was kept under fluorescent light for 4 days at room temperature in a quartz tube. The NMR spectrum was again obtained to confirm cis to trans isomerization. Aliquots of the reaction solution was diluted with 1% TFA and further analyzed by analytical HPLC as described above. The same isomerization reaction was carried out in the case of compound **2**.

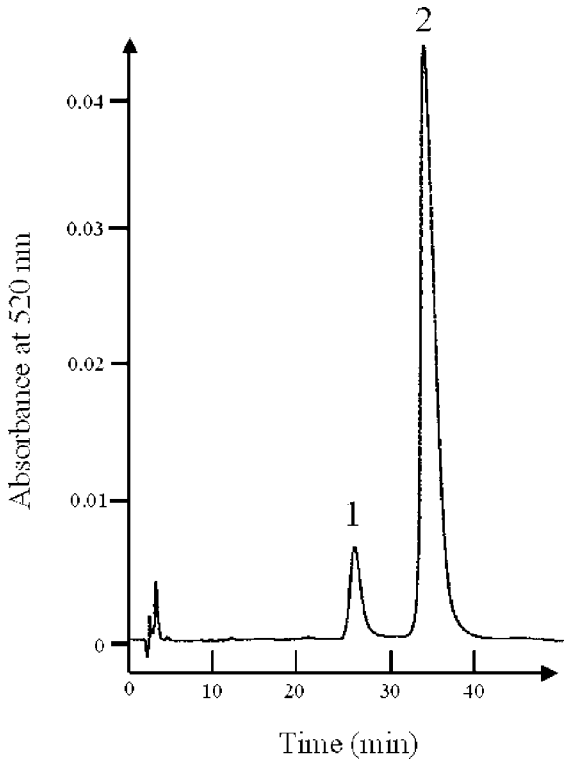

Figure 2. HPLC chromatogram of anthocyanins from eggplant peel extract. Peak numbers correspond to **Figure 1**.

Extraction of Anthocyanins in Dark Condition. To confirm both compounds were not artifacts of light irradiation during extraction, anthocyanins were extracted in a refrigerator at 4 °C for 1 min under dark condition with MeOH containing 3% TFA from fleshly prepared eggplant peel, and an aliquot of extract was immediately analyzed by HPLC as described above.

RESULTS

Figure 2 shows the typical HPLC chromatogram of eggplant extract monitored at 520 nm, and two peaks were detected in the extract. Both peaks were isolated by repeated chromatography. The recoveries of these anthocyanins were 12.5 mg for compound **1** and 57 mg for compound **2**. To assign the chemical structure of the anthocyanins, TOF-MS spectra were measured. The MS spectrum of both compounds showed *m*/*z* 919 for the

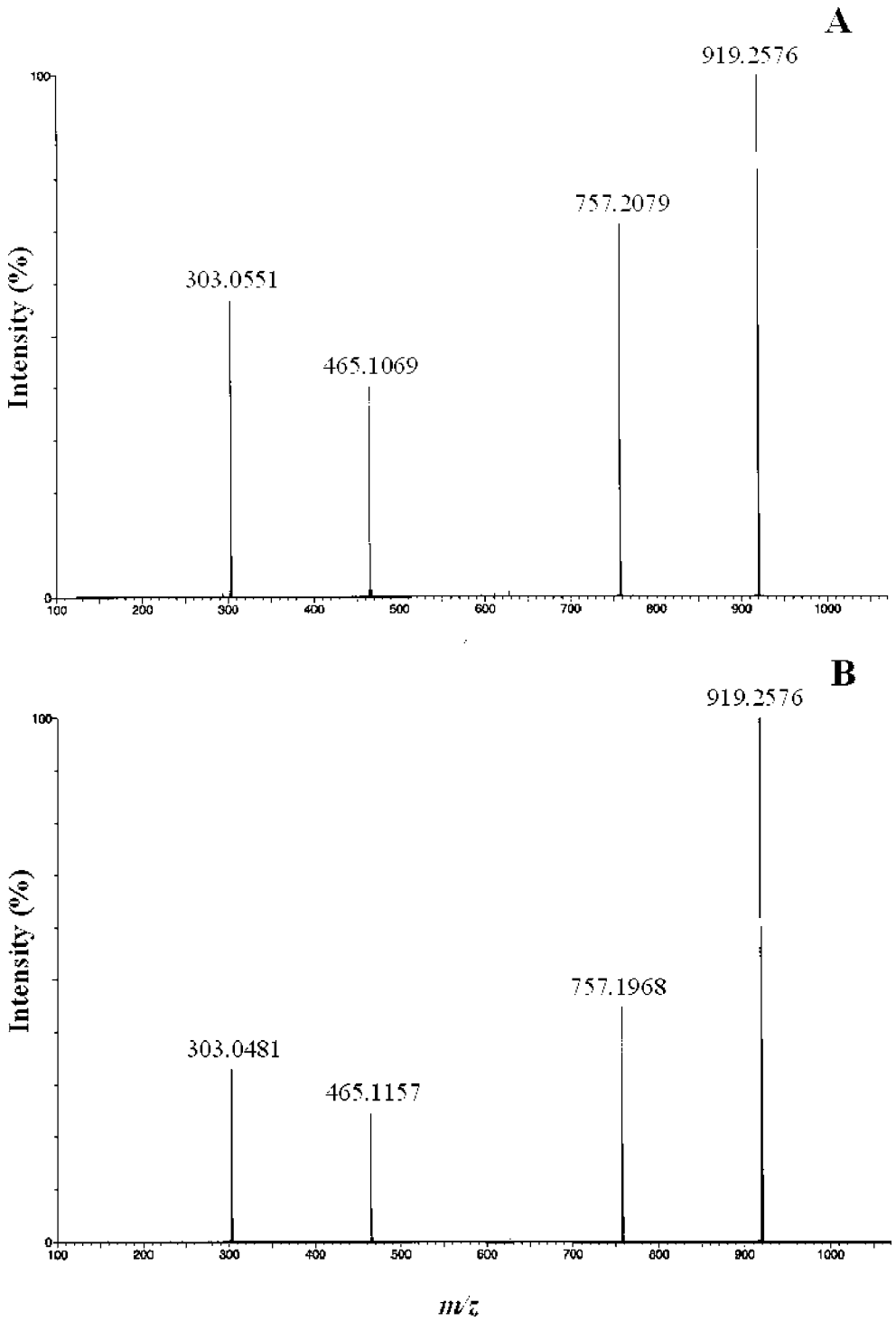

Figure 3. TOF-MSMS spectra of eggplant anthocyanins: (**A**) delphinidin 3-[4-(*cis-p*-coumaroyl)-L-rhamnosyl(1→6)glucopyranoside]-5-glucopyranoside; (**B**) delphinidin 3-[4-(*trans-p*-coumaroyl)-L-rhamnosyl-(1→6)glucopyranoside]-5-glucopyranoside.

molecular ion, in good agreement with the molecular mass of nasunin. Fragment peaks were also observed at *m*/*z* 757 for one hexose loss, at *m*/*z* 465 for *p*-coumaroyl-hexose loss, and at *m*/*z* 303 for the aglycone (delphinidin) (**Figure 3**), indicating that both compounds have delphinidin, *p*-coumaroyl-hexose, and hexose as partial structure. To determine the attached position of the *p*-coumaroyl sugar and hexose on the delphinidin aglycone, ^{1}H and ^{13}C NMR spectra were further employed, and the results are summarized in **Tables 1** and **2**, respectively. The assignments were established by extensive 2D-NMR analyses.

Further structural confirmation was obtained by observing interconversion between compounds **1** and **2** under light illumination. When the solution of compound **1** in CD_3OD/TFA-*d* (9:1) was kept under a fluorescent light for 72 h at room temperature in a quartz tube, an equilibrium mixture of compounds **1** and **2** was formed, the ^{1}H NMR spectrum of which indicated the reaction product was the mixture of compounds **1** and **2** with a ratio of 5/2. The HPLC chromatogram of the equilibrium mixture is shown in **Figure 4**. Because compound **1** was converted to compound **2** by light irradiation, nasunin was extracted in the dark at 4 °C and was immediately analyzed by HPLC to confirm the effect of light on sample preparation. The HPLC pattern obtained was exactly the same as that of **Figure 2**, indicateding that compound **2** is not an artifact

Table 1. ^{1}H NMR (δ, J in Hertz) Spectroscopic Data for Compounds **1** and **2**

position	1	2
delphinidin		
4	8.83 (s)	8.85 (s)
6	6.96 (d, 2)	6.97 (d, 2)
8	6.94 (d, 2)	6.96 (d, 2)
2′	7.71 (s)	7.71 (s)
3-*O*-glucosyl		
1	5.18 (d, 7.8)	5.20 (d, 7.7)
rhamnosyl		
1	4.66 (brs)	4.70 (brs)
4	4.81 (t, 9.5)	4.90 (t, 9.7)
6	0.86 (d, 6)	1.00 (d, 6)
5-*O*-glycosyl		
1	5.56 (d, 8)	5.55 (d, 7.7)
p-coumaroyl		
2	7.58 (d, 8.5)	7.39 (d, 8.5)
3	6.71 (d, 8.5)	6.80 (d, 8.5)
Cα	5.57 (d, 12.6)	6.22 (d, 15.8)
Cβ	6.79 (d, 12.6)	7.55 (d, 15.8)

Table 2. ^{13}C NMR Spectroscopic Data for Compounds **1** and **2**

position	1	2
delphinidin		
2	165	164.9
3	146.7	146.6
4	134.1	134.1
5	157.5	157.4
6	106.5	106.4
7	170.5	170.5
8	98.3	98.3
4a	113.8	114.1
8a	157.7	157.7
1′	120.7	120.6
2′	113.8	113.8
3′	148.6	148
4′	146.8	146.9
3-*O*-glycosyl		
1	103.7	103.6
2	75.7[a]	75.8[a]
3	79.0[b]	79.0[b]
4	72.1[c]	72.3[c]
5	78.4	78.6
6	67.6	68.1
rhamnosyl		
1	102.6	102.9
2	71.9	71.9
3	71.3	71.3
4	75.9	76.3
5	68.5	68.7
6	18.6	18.7
5-*O*-glycosyl		
1	102.6	102.9
2	75.3[a]	73.3[a]
3	79.6[b]	79.6[b]
4	73.0[c]	73.0[c]
5	78.8	78.9
6	63.1	63
p-coumaroyl		
1	128.5	128
2	134.8	132.2
3	116.8	117.8
4	161.3	162.2
Cα	117.2	116.4
Cβ	146.7	148.5
COO	168.7	170

[a–c] Assignment may be interchanged in each column.

produced during extraction and isolation. Conversion from compound **2** to compound **1** also occurred under light illumination (data not shown).

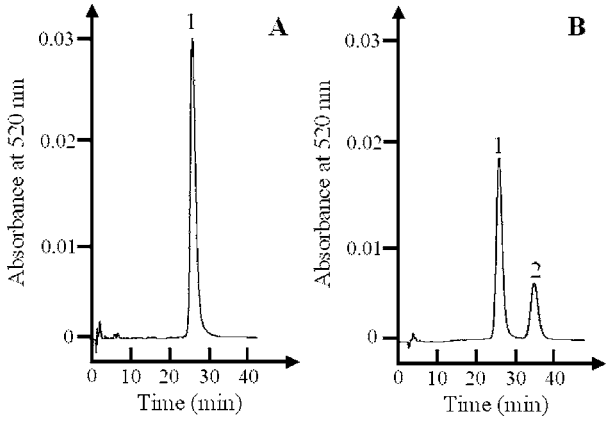

Figure 4. HPLC chromatogram of compound **1** before (**A**) and after (**B**) 72 h of light irradiation.

Taking all of the information obtained above into consideration, it was concluded that the two anthocyanins isolated from eggplant extract were delphinidin 3-[4-(*trans-p*-coumaroyl)-L-rhamnosyl-(1→6)glucopyranoside]-5-glucopyranoside (compound **2**) and delphinidin 3-[4-(*cis-p*-coumaroyl)-L-rhamnosyl-(1→6)glucopyranoside]-5-glucopyranoside (compound **1**), respectively.

DISCUSSION

Anthocyanins have a variety of physiological functions including antioxidant activity (*9*−*18*). Normally, anthocyanins are rather stable in an acid environment as flavilium cation forms (*29*), but they are quite unstable and quickly degraded in neutral to basic conditions, because they are converted into pseudobase to quinoidal structures. However, acylated anthocyanins were suggested to be more stable than nonacylated anthocyanins. Intermolecular stacking between anthocyanins is one of the well-known stabilizing mechanisms of anthocyanins. In the case of acylated anthocyanins, intramolecular stacking between the organic acid and the anthocyanidin moieties are also reported (*28*). Acylated anthocyanins are contained in not only a variety types of flowers but also certain types of edible plants such as purple sweet potato (*6*), red radish (*5*), perilla (*8*), and eggplant (*34*, *35*). They thus have attracted much attention for their health benefits as colored food ingredients (*30*−*38*). Yoshida et al. (*8*) reported the *cis-p*-coumaroyl and *cis*-caffeoyl attached anthocyanins in purple leaves of perilla in which anthocyanin in the trans configuration was preferred. Eggplant is one of the best known anthocyanin-containing vegetables. In the present study, we identified two anthocyanins in eggplant and found that nasunin is the cis−trans isomers of the *p*-coumaroyl moiety of delphinidin 3-[4-(*p*-coumaroyl)-L-rhamnosyl-(1→6)glucopyranoside]-5-glucopyranoside (**Figure 1**). Kuroda et al. previously isolated (*33*) and reported (*34*) the chemical structure of eggplant anthocyanin as nasunin (delphinidin 3-[4-(*p*-coumaroyl)-L-rhamnosyl-(1→6)glucopyranoside]-5-glucopyranoside). However, they did not discuss the stereoisomers.

Figure 2 shows the typical HPLC chromatogram of eggplant extract monitored at 520 nm, and two peaks were detected in the extract. To determine the chemical structures of these anthocyanins, both peaks were isolated by repeated chromatography. To assign the chemical structure of the anthocyanins, TOF-MS spectra were measured. The MS spectrum of compound **1** showed *m*/*z* 919 for the molecular ion, in good agreement with the molecular mass of nasunin. Fragment peaks were also observed at *m*/*z* 757 for one hexose loss, at *m*/*z* 465 for *p*-coumaroyl-hexose loss, and at *m*/*z* 303 for the aglycone

(delphinidin) (**Figure 3A**), indicating that compound **1** has delphinidin, *p*-coumaroyl-hexose, and hexose as partial structure. On the other hand, compound **2** also showed the same molecular ion and fragment peak pattern in its MS spectrum (**Figure 3B**). Thus, to determine the attached position of the *p*-coumaroyl sugar and hexose on the delphinidin aglycone, ^{1}H and ^{13}C NMR spectra were further employed.

The ^{1}H and ^{13}C NMR spectrum of both compounds showed the presence of a delphinidin moiety, a *p*-coumaroyl moiety, and three sugar moieties, including a rhamnosyl and two glucosyl moieties. The ^{1}H NMR of compound **1** also revealed signals at lower field similar to those of compound **2**, except for the observation of a pair of olefinic signals with cis-coupling [δ 5.57 and 6.79 (each 1H, d, $J = 12.6$ Hz)], suggesting that the *p*-coumaroyl group in compound **1** has a cis configuration. Furthermore, the triplet proton signal at δ 4.81 ($J = 9.5$ Hz) in the ^{1}H NMR of compound **1**, which appeared at lower field, was assigned to the rhamnosyl H-4 by 2D-NMR analyses, indicating the location of the *cis*-*p*-coumaroyl group was the same as that of compound **2**. Further structural confirmation was obtained by the formation of compound **1** from compound **2** by fluorescent lamp. When the solution of compound **1** in CD_3OD/TFA-*d* (9:1) was kept under a fluorescent room lamp for 72 h at room temperature, an equilibrium mixture of compounds **1** and **2** was formed, the ^{1}H NMR spectrum of which indicated the reaction product was the mixture of compounds **1** and **2** with a ratio of 5/2. The HPLC chromatogram of the equilibrium mixture is shown in **Figure 4**. The same conversion was observed when compound **2** was irradiated by fluorescent lamp (data not shown). Because compounds **1** and **2** were found to be interconvertible with light irradiation, nasunin was extracted in the dark at 4 °C and was immediately analyzed by HPLC. The HPLC pattern obtained was the same as that of **Figure 2**. Thus, it was confirmed that neither compound **1** nor compound **2** determined in the present study was an artifact produced during the extraction and isolation processes but existed originally in the eggplant.

Finally, it was concluded that the two anthocyanins isolated from eggplant extract were delphinidin 3-[4-(*trans*-*p*-coumaroyl)-L-rhamnosyl-(1→6)glucopyranoside]-5-glucopyranoside (compound **2**) and delphinidin 3-[4-(*cis*-*p*-coumaroyl)-L-rhamnosyl(1→6)glucopyranoside]-5-glucopyranoside (compound **1**), respectively. Stereoisomers of anthocyanins have been reported by Hosokawa et al. for cyanidin (*25*) or pelargonidin (*26*) on the basis of acylated anthocyanins in *Hyacinthus orientalis*. However, there has been no report published about the stereoisomer of delphinidin-based acylated anthocyanins in food materials. It is known that a structural difference significantly affects the in vivo behaviors of food components, including their uptake and metabolism (*21*−*24*, *39*−*42*). Thus, it will be interesting to compare the health benefits of stereoisomers of the eggplant anthocyanins found in the present study. Further studies are in progress to clarify these points.

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