Ultraviolet-absorbing Substances in the Translucent Bracts of Davidia involucrata (Davidiaceae)

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Abstract Ultraviolet-absorbing substances in the bracts of *Davidia involucrata*, which is endemic to China, were surveyed. Two large translucent bracts of the species put on the inflorescence such as a parasol and selectively absorbed UV radiation, though visible light almost completely transmitted. It was shown by high performance liquid chromatography (HPLC) that much amounts of UV-absorbing substances are accumulated. Seven flavonol glycosides were isolated by various chromatographic techniques such as paper, column and HPLC, and identified as quercetin 3-*O*-galactoside, quercetin 3-*O*-glucoside, quercetin 3-*O*-glucoside, quercetin 3-*O*-glucoside, kaempferol 3-*O*-glalactoside, quercetin 3-*O*-(2″-acetylglalactoside) and quercetin 3-*O*-(acetylglucoside) by UV spectral survey, LC-MS, ¹H and ¹³C NMR, acid hydrolysis, and direct TLC and HPLC comparisons with authentic samples. Thus, it was proved that the flavonol glycosides, especially quercetin 3-*O*-glalactoside and 3-*O*-glucoside, act as the important UV shields in the translucent bracts of *D. involucrata* and protect the inflorescence from noxious UV radiation.

Key words: Davidiaceae, *Davidia involucrata*, Flavonols, Quercetin 3-O-glycosides, UV shields.

Introduction

Ultraviolet radiation is noxious for all organisms including human. The plants, especially green plants, are directly exposed to not only visible light but also UV radiation. Photosynthetically active radiation penetrates effectively into the mesophyll and is attenuated by as little as 20 to 30% as it passes through the epidermis (Caldwell et al., 1983). Nuclei are organelles where primary lesions can result from UV radiation, especially UV-B (Brandle et al., 1977; Howland, 1975). Generally, radiation should be selectively filtered to remove the short wavelength UV component before it reaches the nuclei and chloroplasts. The wavelength selectivity of absorption in the epidermis of most plant species is much more pronounced and can often be attributed to

flavonoids and related UV-absorbing substances (Robberecht and Caldwell, 1978). Since many of the UV-absorbing compounds of plant cells are contained within the vacuole (McClure, 1975), this is the site for much of the UV absorption in the epidermis. Thus, flavonoid compounds as UV shields have been reported from various plant species, e.g., quercetin 3-O-(3",6"-p-coumaroylglucoside) and kaempferol 3-*O*-(3",6"-*p*coumaroylglucoside) from Pinus sylvestris L. (Pinaceae) (Jungblut et al., 1995), quercetin 3-Ogalactoside, myricetin 3-O-rutinoside and two pcoumaroyl kaempferol 3-O-glucosides from Quercus ilex L. (Fagaceae) (Skaltsa et al., 1994), and quercetin, apigenin and luteolin glycosides from Olea europaea L. (Oleaceae) (Karabourniotis et al., 1992).

Recently, eight flavonol glycosides were isolat-



Fig. 1. The bracts of *Davidia involucrata*, and UV-A and UV-B contents (w/m²) in sunshine before and after passing the translucent bract.

ed as UV-absorbing substances from the translucent bracts of Rheum nobile Hook. f. et Thomson (Polygonaceae), which grows in the alpine zone of the eastern Himalayas (Iwashina et al., 2004). Their flavonoids were identified as quercetin 3-O-[6"-(3-hydroxy-3-methylglutaroyl)-glucoside], quercetin 3-O-glucoside, quercetin 3-O-galactoside, quercetin 3-O-rutinoside, quercetin 3-Oarabinoside, quercetin itself, quercetin 7-O-glycoside and kaempferol glycoside. Of their compounds, the former five glycosides were major ones and have been shown to be the important UV shields in this species (Iwashina et al., 2004). Quercetin 3-O-[6"-(3-hydroxy-3-methylglutaroyl)-glucoside] was a new acylated flavonol glycoside.

Saussurea involucrata Karel. et Kir. (Asteraceae) also put the large translucent cream-colored bracts concealing the huge inflorescence and grows in the alpine zone of central Asia such as Kyrgyz, Kazakhstan, Mongolia and China. From their bracts and also leaves, much amount of quercetin 3-*O*-rutinoside, which may be acts as UV shields, has been isolated together with four minor flavone glycosides, apigenin, luteolin, hispidulin and nepetin 7-*O*-glucosides (Kusano *et al.*, 2007).

Davidia involucrata Baill. is endemic to southwestern China (Mabberley, 1997) and is sometimes cultivated in the gardens and parks as ornamentals. We noticed that the two translucent bracts, which bear in spring, put on the inflorescence such as a parasol, and presumed that UVabsorbing substances, *e.g.*, flavonoids, are accumulated in the bracts such as *Rheum nobile* and *Saussurea involucrata*.

The flavonoid compounds have been isolated from the leaves of *D. involucrata* and identified as quercetin 3-*O*-glucoside, quercetin 3-*O*-galactoside, quercetin 3-*O*-arabinoside and kaempferol 3-O-galactoside (Iwashina and Hatta, 1998). Ouyang and Zhou (2003) have been surveyed the leaves for flavonoids and isolated kaempferol, kaempferol 3-O-glucoside and quercetin in addition to the above flavonoids. Of their compounds, quercetin 3-O-galactoside has also been found in the bracts as a major flavonoid, together with minor quercetin 3-O-rhamnoside (Rast, 1968).

In this paper, we describe the identification of the flavonoid glycosides in the bracts and function of their compounds as UV filter.

Materials and Methods

Plant materials

Davidia involucrata Baill. is cultivated in the Tsukuba Botanical Garden, National Museum of Nature and Science, Tsukuba, Japan (Acc. no. TBG 43668). The fresh bracts were collected in May, 2004.

Measurement of UV radiation in sunshine, and direct UV-visible spectral survey of bracts

UV-A (355 nm) and B (313 nm) radiation in sunshine before and after passing the translucent bract was measured by UV monitor MS-211-I (EKO Co. Ltd.). UV-visible spectra of the fresh bracts were surveyed on a Shimadzu MPS-2000 multi purpose recording spectrophotometer between 300 and 700 nm.

Qualitative and quantitative HPLC of crude extract and isolated UV absorbing substances

Qualitative HPLC survey of the crude extracts from the bracts and the isolated UV-absorbing substances were performed with Shimadzu HPLC systems using a Shim-pack CLC-ODS (I.D. 6.0×150 mm; Shimadzu) at flow-rate: 1.0 ml min^{-1} , detection: 190–700 nm and eluent: MeCN/H₂O/H₃PO₄ (22:78:0.2). Fresh bracts (0.1 g) were extracted with MeOH (2 ml) for quantitative HPLC survey. After keeping for 1 day in room temperature, the extracts were filtered and analysed by HPLC.

Preparative HPLC was performed with Tosoh HPLC systems using a Senshu-pak PEGASIL ODS column (I.D. 10.0×250 mm, Senshu Scientific Co. Ltd.) at flow-rate: 1.5 ml min^{-1} , detection: 350 nm and eluent: HCOOH/MeCN/H₂O (1:20:79).

Liquid chromatograph-mass spectra (LC-MS)

LC-MS was measured using a PEGASIL ODS column (I.D. 2.0×150 mm; Senshu Scientific Co. Ltd.), at flowe-rate of 0.2 ml min^{-1} , eluent: MeCN/H₂O/HCOOH (22:78:1), and ESI⁺ 4.5 kV, ESI⁻ 3.5 kV, 250°C.

Extraction and isolation of UV absorbing substances

Fresh bracts (ca. 39 g) were extracted with MeOH. After evaporation, the extracts were applied to preparative paper chromatography (PPC) using solvent systems: BAW (n-BuOH/HOAc/H₂O=4:1:5, upper phase), 15%HOAc and then BEW $(n-BuOH/EtOH/H_2O=$ 4:1:2.2). The isolated compounds were finally purified by Sephadex LH-20 column chromatography using solvent system: 70% MeOH. Of seven compounds (1-7) detected by HPLC survey, 1 and 3 were obtained as pure solutions, and 2 and 3, and 5 and 6 as the mixtures, respectively. Compound 4 could not be isolated for the minute amount. For further isolation of minor compounds 5 and 6, fresh bracts (136 g) were collected in May, 2005 and extracted with MeOH. After application to PPC, they were isolated by preparative HPLC and obtained as pale yellow powder (5) and pure solution (6).

Identification of UV absorbing substances

UV absorbing substances were identified by UV spectral survey according to Mabry *et al.* (1970), LC-MS, acid hydrolysis (in 12% aq.HCl, 100°C, 30 min) and characterization of its products, ¹H and ¹³C NMR (in pyridine- d_5 , 500 MHz for ¹H NMR and 125 MHz for ¹³C NMR), direct TLC and HPLC comparisons with authentic samples. TLC, HPLC, LC-MS, and ¹H and ¹³C NMR data of UV absorbing substances are as follows.

Quercetin 3-*O*-sambubioside (1). TLC: Rf 0.42 (BAW), 0.42 (BEW), 0.47 (15% HOAc);



Fig. 2. UV-visible spectral curve of *Davidia involucrata* bract.

UV-dark purple, UV/NH₃-yellow. HPLC: Rt (min) 4.38. UV: λmax (nm) MeOH 256, 267 sh, 357; +NaOMe 272, 328, 406 (inc.); +AlCl₃ 274, 432; +AlCl₃/HCl 269, 298, 361, 397; +NaOAc 273, 326, 388; +NaOAc/H₃BO₃ 262, 296, 378.

Mixture of quercetin 3-*O*-galactoside (hyperin, **2**) and quercetin 3-*O*-glucoside (isoquercitrin, **3**). TLC: Rf 0.50 (BAW), 0.51 (BEW), 0.22 (15% HOAc); UV-dark purple, UV/NH₃-yellow. HPLC: Rt (min) 5.25 (**2**) and 5.52 (**3**). UV: λ max (nm) MeOH 256, 267 sh, 358; +NaOMe 272, 329, 409 (inc.); +AlCl₃ 275, 435; +AlCl₃/HCl 269, 299, 363, 400; +NaOAc 274, 325, 389; +NaOAc/H₃BO₃ 262, 297, 378.

Quercetin 3-*O*-arabinoside (4). TLC: Rf 0.63 (BAW), 0.57 (BEW), 0.12 (15% HOAc); UV-dark purple, UV/NH₃-yellow. HPLC: Rt (min) 5.96. UV: λ max (nm) MeOH 256, 270 sh, 358; +NaOMe 273, 329, 407 (inc.); +AlCl₃ 274, 421; +AlCl₃/HCl 268, 299, 362, 398; +NaOAc 273, 324, 390; +NaOAc/H₃BO₃ 261, 296, 377.

Quercetin 3-O-(2"-acetylgalactoside) (6). TLC: Rf 0.71 (BAW), 0.69 (BEW), 0.43 (15% HOAc); UV-dark purple, UV/NH₃-yellow. HPLC: Rt (min) 8.86. UV: λ max (nm) MeOH 256, 267 sh, 356; +NaOMe 273, 329, 407 (inc.); +AlCl₃ 274, 431; +AlCl₃/HCl 270, 298, 359, 397; +NaOAc 273, 325, 394; +NaOAc/H₃BO₃

262, 297, 373. LC-MS: m/z 477 [M+H]⁺ and m/z 475 [M-H]⁻. ¹H NMR (500 MHz, pyridine d_5): δ 13.43 (1H, s, 5-OH), 8.35 (1H, d, J=2.1 Hz, H-2'), 8.20 (1H, dd, J=2.1 and 8.4 Hz, H-6'), 7.30 (1H, d, J=8.6 Hz, H-5'), 6.71 (1H, d, J=1.8 Hz, H-8), 6.62 (1H, d, J=2.1 Hz, H-6), 6.29 (1H, d, J=7.6 Hz, galactosyl H-1), 6.27 (1H, d, J=6.7 Hz, galactosyl H-2), 4.64 (1H, d, J=2.7 Hz, galactosyl H-4), 4.39 (1H, m, galactosyl H-6a), 4.38 (1H, m, galactosyl H-3), 4.27 (1H, dd, J=5.8 and 11.0 Hz, galactosyl H-6b), 4.18 (1H, t, J=12.5 Hz, galactosyl H-5), 2.16 (3H, s, acetyl CH₃). ¹³C NMR (125 MHz, pyridine- d_5): δ (quercetin) 157.4 (C-2), 134.9 (C-3), 178.5 (C-4), 162.9 (C-5), 99.7 (C-6), 165.7 (C-7), 94.4 (C-8), 157.4 (C-9), 105.3 (C-10), 123.1 (C-1'), 116.3 (C-2'), 146.8 (C-3'), 150.7 (C-4'), 117.4 (C-5'), 122.3 (C-6'); δ (galactose) 101.6 (C-1), 74.4 (C-2), 73.1 (C-3), 69.8 (C-4), 77.6 (C-5), 61.5 (C-6); δ (acetic acid) 170.7 (COO–), 21.4 (CH₃).

Quercetin 3-*O*-(acetylglucoside) (7). TLC: Rf 0.71 (BAW), 0.69 (BEW), 0.43 (15% HOAc); UV – dark purple, UV/NH₃ – yellow. HPLC: Rt (min) 9.17. UV: λ max (nm) MeOH 256, 266 sh, 356; +NaOMe 273, 329, 407 (inc.); +AlCl₃ 274, 431; +AlCl₃/HCl 270, 298, 359, 397; +NaOAc 273, 325, 394; +NaOAc/H₃BO₃ 262, 297, 373. LC-MS: *m/z* 477 [M+H]⁺ and *m/z* 475 [M-H]⁻.

Results and Discussion

Absorption of UV radiation with the translucent bracts

When the amount of UV radiation (UV-A and UV-B) in sunshine were measured before and after passing the bracts, both the amount of UV-A and UV-B clearly decreased in the latter case (Fig. 1). Direct UV-visible spectra of the translucent bracts are shown in Fig. 2. Though weak absorption maxima were present in 675 and 480 sh nm in visible range (400–700 nm), showing the presence of a very small amount of chlorophylls, other absorption maxima could not be detected. On the other hand, UV range (300–400 nm) was

strongly absorbed. Thus, it was shown that the translucent bracts of *D. involucrata* selectively absorb UV radiation, but visible light is almost completely transmitted, so that we presumed that the epidermis of the bracts contain much amount of UV-absorbing substances which are present in the cells to screen UV radiation.

Identification of UV-absorbing substances in the bracts

HPLC patterns of the translucent bracts were monitored by 280 nm (UV-B range) (Fig. 3). Some peaks appeared on the chromatogram and were presumed to be flavonoid compounds by



Fig. 3. HPLC pattern of MeOH extracts from the bracts of *Davidia involucrata*. 1=quercetin 3-O-sambubioside, 2=quercetin 3-O-galactoside, 3=quercetin 3-O-glucoside, 4=quercetin 3-O-arabinoside, 5=kaempferol 3-O-galactoside, 6=quercetin 3-O-(2"-acetylgalactoside), and 7=quercetin 3-O-(acetylglucoside).

UV spectral properties. Major compounds 2 and 3 were obtained as the mixture. UV spectral survey according to Mabry et al. (1970) showed that they are flavonol glycosides having free 5-, 7-, 3'- and 4'-hydroxyl and a substituted 3-hydroxyl groups. By acid hydrolysis, they liberated quercetin, glucose and galactose. From the results described above, they were presumed to be the mixture of the glycosides which attached galactose or glucose to 3-position of quercetin. Finally, they were identified as the mixture of quercetin 3-O-galactoside (2, Fig. 5) and quercetin 3-O-glucoside (3, Fig. 6) by direct TLC and HPLC comparisons with authentic hyperin and isoquercitrin from the flowers of Astrophytum ornatum (DC.) Web. (Cactaceae) (Iwashina and Ootani, 1986) and the fronds of Cyrtomium falcatum (L. f.) C. Presl (Dryopteridaceae) (Iwashina et al., 2006). Quercetin 3-O-galactoside and 3-O-glucoside have been isolated from



Fig. 4. Quercetin 3-O-samububioside (1).

Table 1. Quantitative HPLC analysis of UV-absorbing substances in the bracts of Davidia involucrata

Values	1	2 and 3	4	5	6	7
Area	225,972	9,623,359	1,701,153	174,864	1,846,709	498,730
Fresh weight* (mg/g)	0.09	3.65	0.65	0.07	0.70	0.19
Ratio	1.6%	68.5%	12.1%	1.2%	13.1%	3.5%

Fresh bract (0.1 g) was extracted with MeOH (2 ml). Measurement was 280 nm.

*Calculated from the absorption coefficient of authentic quercetin 3-O-galactoside.

1=quercetin 3-O-sambubioside, 2=quercetin 3-O-galactoside, 3=quercetin 3-O-glucoside, 4=quercetin 3-O-arabinoside, 5=kaempferol 3-O-galactoside, 6=quercetin 3-O-(2''-acetylgalactoside) and 7=quercetin 3-O-(acetylglucoside).



Fig. 5. Quercetin 3-O-galactoside (hyperin, 2).



Fig. 6. Quercetin 3-O-glucoside (isoquercitrin, 3).

the leaves of this species (Iwashina and Hatta, 1998).

It was shown by UV spectral survey that compound 1 is also 3-O-substituted 5,7,3',4'-tetrahydroxyflavone. Quercetin as an aglycone, and xylose and glucose as glycosidic sugars were produced by acid hydrolysis. Thus, compound 1 was characterized as quercetin 3-O-xylosylglucoside. Finally, the compound was completely identified as quercetin 3-O-xylosyl-(1 \rightarrow 2)-glucoside, *i.e.*, quercetin 3-O-sambubioside (Fig. 4) by direct TLC and HPLC comparison with authentic sample from the leaves of *Aucuba japonica* Thunb. (Cornaceae) (Iwashina *et al.*, 1997). Quercetin 3-O-sambubioside has been reported from *D. involucrata* for the first time.

Acid hydrolysis of compound **4** liberated quercetin and arabinose. Since UV spectral survey of the original glycoside showed the presence of free 5-, 7-, 3'- and 4'-hydroxyl groups, it was determined that arabinose is attached to 3-posi-



Fig. 7. Quercetin 3-O-arabinoside (4).

tion of quercetin. By direct TLC and HPLC comparison with authentic sample, which has been isolated from the bracts of *Rheum nobile* (Iwashina *et al.*, 2004), compound **4** was shown to be quercetin 3-*O*-arabinoside (Fig. 7). The compound has already been detected from the leaves of this species (Iwashina and Hatta, 1998; Ouyang and Zhou, 2003).

Though minor compound **5** could not be isolated for small amount, it was presumed to be kaempferol derivative by UV spectral properties (appearance of λ max 266 and 345 nm) on HPLC. It was identified as kaempfeol 3-*O*-galactoside (Fig. 8) by HPLC comparison with authentic trifolin from the leaves of *Cornus canadensis* L. (Cornaceae) (Iwashina and Hatta, 1993). Kaempferol 3-*O*-galactoside has also been reported from *D. involucrata* (Iwashina and Hatta, 1998; Ouyang and Zhou, 2003).

Compound **6** was obtained by pale yellow powder. It was shown to be 3-*O*-substituted quercetin by UV spectral survey according to Mabry *et al.* (1970). Quercetin and galactose was liberated by acid hydrolysis. However, its retention time does not agreed with that of authentic hyperin. The molecular ion peaks, m/z 477 $[M+H]^+$ and m/z 475 $[M-H]^-$, showing the attachment of each 1 mol galactose and acetic acid to quercetin, appeared on the chromatogram by LC-MS survey. In ¹H NMR spectrum, a singlet (δ 2.16) corresponding to acetyl CH₃ appeared with five aromatic protons (H-6, H-8, H-2', H-5' and H-6') and a galactosyl anomeric proton (δ 6.29, *J*=7.6 Hz). The attachment of acetyl group



Fig. 8. Kaempferol 3-O-galactoside (trifolin, 5).



Fig. 9. Quercetin 3-O-(2"-acetylgalactoside) (6).

to 2-position of galactose was proved by ¹³C NMR spectrum, *i.e.*, the diagnostic shift to lower field of carbon signal (δ 74.4) corresponding to galactosyl C-2 (Agrawal, 1989). Moreover, it was confirmed by ¹H-¹H COSY, FG-HSQC and FG-HMBC (Fossen and Andersen, 2006). Thus, compound 6 was completely identified as quercetin $3-O-\beta-D-(2''-acetylgalactopyranoside)$ (Fig. 9). The rare acylated flavonoid has only been reported from the whole plants of Hypericum perforatum L. (Guttiferae) (Jürgenliemk and Nahrstedt, 2002) and similar acylated quercetin glycosides, quercetin 3-O-(3"-acetylgalactoside) and quercetin 3-O-(6"-acetylgalactoside), have been found in Ledum palstre L. (Ericaceae) (Harborne and Baxter, 1999).

LC-MS survey of compound 7 indicated m/z 477 [M+H]⁺ and m/z 475 [M-H]⁻, showing the attachment of each 1 mol hexose and acetic acid

to quercetin. TLC, LC-MS and UV spectral data showed that 7 is quercetin 3-*O*-(acetylhexoside), so that we presumed this compound as to be quercetin 3-*O*-(acetylglucoside). Complete identification of the compound is now in progress.

Quantitative HPLC survey of the bract is shown in Table 1. Of their flavonoids isolated from the bracts, major ones were quercetin 3-Ogalactoside (2) and 3-O-glucoside (3). Their relative amount was 68.5% (3.65 mg/g fresh bract). On the other hand, other flavonoids (1, 4–7) were between 13.1–1.2% (0.70–0.07 mg/g).

UV-absorbing substances in the bracts of D. involucrata were all flavonol glycosides, especially quercetin 3-O-glycosides. Quercetin glycosides have been reported as the predominant flavonoid in response to UV-B radiation in some plants species, e.g., Anethum graveolens L. (Umbelliferae) (Möhle et al., 1985), Brassica napus L. (Brassicaceae) (Olsson et al., 1998; Wilson Greenberg, 1993), Petunia and hvbrida (Solanaceae) (Ryan et al., 1998, 2002), Trifolium repens L. (Leguminosae) (Hofmann et al., 2000) and Betula pendula Roth (Betulaceae) (Lavola et al., 1997). Petunia and wild-type of Arabidopsis thaliana L. leaves exposed to low UV-B conditions contained predominantly kaempferol glycosides, with low levels of quercetin glycosides (Ryan et al., 1998, 2001). However, when the leaves were treated with UV-B, an increase of quercetin was observed. These results suggest that quercetin glycosides protect the plants from UV-B damage. Recently, it was suggested that quercetin glycoside function as UV protecting substances in the translucent bracts of Rheum nobile, which grows in the alpine zone of the Himalayas (Iwashina et al., 2004). In also the case of Davidia involucrata, quercetin glycosides predominantly occurred in the bracts. Thus, it was shown that quercetin glycosides, especially quercetin 3-O-galactoside and quercetin 3-O-glucoside, mainly act as UV shields in the translucent bracts of D. involucrata, and protect the inflorescence from noxious UV radiation. More recently, it has been reported that the bracts of D. involucrata contain much amounts of flavonoids,

which were not qualitatively characterized, and act as pollination attractants to insects, especially Hymenoptera (Hu *et al.*, 2007), so that the bracts may be functional for both pollination attractants and UV shields.

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