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Flavonoid 5-glucosides from the cocoon shell of the silkworm, *Bombyx mori*

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Abstract

The flavonoid 5-glucosides, quercetin 5,4′-di-*O*-β-D-glucopyranoside and quercetin 5,7,4′-tri-*O*-β-D-glucopyranoside, together with the known quercetin 5-*O*-β-D-glucopyranoside, were isolated from the cocoon shell of the silkworm, *Bombyx mori*. The structures were identified by spectroscopic analysis. These flavonoid glucosides were not present in mulberry leaves, the silkworm's only food, and they are considered to be metabolites produced by the silkworm. © 2002 Published by Elsevier Science Ltd.

Keywords: *Bombyx mori*; Silkworm; Cocoon; Flavonoid; Quercetin 5,4′-di-*O*-β-D-glucopyranoside; Quercetin 5,7,4′-tri-*O*-β-D-glucopyranoside; Quercetin 5-*O*-β-D-glucopyranoside

1. Introduction

The silkworm, *Bombyx mori,* is a monophagous insect whose only food is mulberry leaves. The cocoon shell of the silkworm consists mainly of proteins such as fibroin and sericin. In addition to these proteins, the cocoon shell contains small amounts of pigments, waxes and carbohydrates. Flavonoids have been found as pigments in the cocoon shells of some silkworm races (Oku, 1934; Fujimoto and Kawakami, 1958; Hayashiya et al., 1959). In these previous studies, qualitative analyses of flavonoids in cocoon shells have been based on color reactions, whereas the chemical structures of flavonoids remain to be determined. Recently, metabolic conversion processes of quercetin and its glucosides by mammals have been postulated (Day et al., 2000; Gee et al., 2000). However, the metabolic processes of insects have not been well studied. We have identified three flavonoid 5-glucosides including two novel flavonoid glucosides from the cocoon shell of the silkworm race "Multi-Bi" of *B. mori*. Because these compounds are not present in the mulberry leaf diet, they are considered to be metabolites of *B. mori*.

2. Results and discussion

Three pigments were detected by HPLC from the pigment extract of the cocoon shell of Multi-Bi. They were isolated and identified as follows: The UV-vis spectrum of the purified compound **1** in MeOH showed absorption maxima at 251 and 359 nm. The spectral changes induced by the various shift reagents suggested that compound **1** was a flavonoid having two free hydroxyl groups at C-7 and at C-3, and no free hydroxyl group at C-4′ (Markham, 1982). The FT–IR spectrum showed the presence of an OH group and C–O–C linkage at 3370 and 1074 cm^{-1}, respectively. The flavonoid aglycone in compound **1** obtained by hydrolysis with β-glucosidase was purified by preparative HPLC. Its UV-vis spectrum, ^{1}H and ^{13}C NMR spectroscopic data in DMSO-d_6 were consistent with those of quercetin (Markham and Geiger, 1994; Markham et al., 1978). Therefore, the aglycone of compound **1** was identified as quercetin. When the sugar moiety of compound **1** was submitted to silica gel 60 HPTLC, after spraying, one blue spot of an aldohexose was observed at an R_f value, the same as that of D-glucopyranose. The FD–MS spectrum indicated a relative molecular mass of 626, which is compatible with a quercetin diglycoside ($C_{27}H_{30}O_{17}$). The ^{1}H NMR spectrum of compound **1** was compatible with those of a quercetin derivative.

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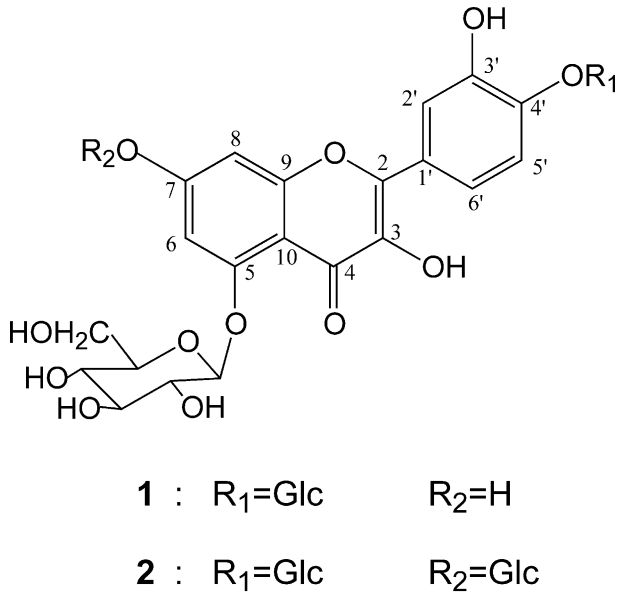

Furthermore, the spectrum indicated the sugar moiety was a D-glucoside, and the signals at 4.80 ppm (1 H, *d*, $J=7.2$ Hz) and 4.84 ppm (1 H, *d*, $J=7.2$ Hz) indicated two β linkages. In the ^{13}C NMR spectrum, the C-4 signal was shifted 4.1 ppm upfield compared with quercetin, indicating the presence of a glucoside at the C-5 hydroxyl group (Markham et al., 1978). This was confirmed from the NOESY correlation between the H-6 (δ 6.77 ppm) and the anomeric glucosyl H-1 (δ 4.80 ppm). The C-4′ of compound **1** was shifted 1.4 ppm upfield compared with that of quercetin (Markham et al., 1978), indicating that the C-4′ hydroxyl group of compound **1** was glucosylated. The presence of the glucoside at the C-4′ hydroxyl group was confirmed from the NOESY correlation between the H-5′ (δ 7.25 ppm) and the other anomeric glucosyl H-1 (δ 4.84 ppm). From this spectroscopic and chemical evidence, compound **1** was identified as quercetin 5,4′-di-*O*-β-D-glucopyranoside. This was the main flavonoid in the cocoon shell of Multi-Bi.

The UV-vis spectrum of compound **2** in MeOH showed absorption maxima at 252 and 359 nm. The spectral changes induced by the various shift reagents indicated that the flavonoid had a free hydroxyl group at C-3, and no free hydroxyl group at C-7 or at C-4′ (Markham, 1982). The FT–IR spectrum of compound **2** showed the presence of an OH group and C–O–C linkage as described above. The FD-MS spectrum indicated a relative molecular mass of 788, which is compatible with a quercetin trihexoside ($C_{33}H_{40}O_{22}$). The ^1H NMR spectrum of compound **2** was compatible with a quercetin derivative. Three glucosyl anomeric proton signals were indicative of β linkages (δ 5.15 ppm, 1H, *d*, $J=6.5$ Hz; δ 4.91 ppm, 1H, *d*, $J=7.5$ Hz; δ 4.83 ppm, 1H, *d*, $J=7.0$ Hz). The chemical shift of the C-4 (δ 171.8 ppm) signal suggested the presence of a glucoside at the C-5 hydroxyl group (Markham et al., 1978). Furthermore, the proton singlet of the C-5 hydroxyl group of quercetin was absent in the ^1H NMR spectrum of compound **2**. Comparison of the chemical shifts of the H-2′, H-5′ and H-6′ signals with those in **1** (Table 1), the C-4′ hydroxyl group was glucosylated. Furthermore, the C-7 signal of compound **2** was 1.9 ppm upfield compared with that of compound **1**. This suggested that the C-7 hydroxyl group of compound **2** was glucosylated (Markham et al., 1978). From the spectroscopic and chemical evidence, compound **2** was identified as quercetin 5,7,4′-tri-*O*-β-D-glucopyranoside.

Compound **3** was identified as quercetin 5-*O*-β-D-glucopyranoside by comparison of physical data with those in the literature (Glennie and Harborne, 1971). The UV-

Table 1
^{13}C NMR spectral data of compounds **1–3** in DMSO-d_6 (δ: chemical shifts; ppm)

Carbon	1	2	3
Flavonoid moiety			
C-2	142.5	143.0	143.5
C-3	137.7	138.2	137.2
C-4	171.7	171.8	171.7
C-5	158.3	157.8	158.4
C-6	103.2	102.0[g]	103.1
C-7	163.0	161.1	162.4
C-8	97.3	97.9	97.3
C-9	157.2	156.8	157.1
C-10	106.0	107.7	106.3
C-1′	125.2	125.2	122.0
C-2′	114.7	115.0	114.7
C-3′	146.3[e]	146.5[h]	145.0
C-4′	146.2[e]	146.4[h]	147.3
C-5′	115.9	115.9	115.6
C-6′	119.0	119.0	119.5
5-O-Glucoside			
C-1	103.8	103.0[g]	103.9
C-2	73.5	73.6	73.7
C-3	75.7[d]	75.8[i]	75.7
C-4	69.6[e]	69.7[j]	69.7
C-5	77.4[f]	77.4[k]	77.5
C-6	60.6	60.8[l]	60.8
4′-O-Glucoside			
C-1	101.4	101.5[g]	
C-2	73.1	73.2	
C-3	75.4[d]	75.7[i]	
C-4	69.5[e]	69.5[j]	
C-5	77.1[f]	77.2[k]	
C-6	60.6	60.6[l]	
7-O-Glucoside			
C-1		99.3	
C-2		73.0	
C-3		76.4[i]	
C-4		69.7[j]	
C-5		77.0[k]	
C-6		60.7[l]	

[e–l] Corresponding assignments may be reversed.

vis, ^{1}H and ^{13}C NMR spectroscopic data of compound **3** is shown in Table 1 and Experimental.

The same three flavonoid 5-glucosides in the cocoon shell of Multi-Bi were also found in two other cocoon races, namely the white cocoon shell of BC 25 and the pale yellow cocoon shell of Daizo (G), based on UV-vis spectra and the retention times on HPLC (data not shown).

The presence of a glycoside at the C-5 hydroxyl group of a flavonoid is very rare. This absence is presumably due to the fact that such glycosylation involves disruption of the strong hydrogen bonding between the C-5 hydroxyl group and the 4-keto group (Harborne, 1967). Although quercetin 5-*O*-β-D-glucopyranoside has been found in some plants (Harborne, 1967; De Israilev et al., 1991), quercetin 5,4′-di-*O*-β-D-glucopyranoside and quercetin 5,7,4′-tri-*O*-β-D-glucopyranoside have not been reported. On the HPLC evidence, these flavonoid 5-glucosides were not present in the leaves of *Morus alba* L., the food of *B. mori* (data not shown).

Many flavonoids have been identified from the root bark of the mulberry tree (Nonura, 1988). The leaves of *M. alba* L. contain quercetin derivatives such as isoquercitrin and rutin (Naito, 1968). It was reported previously that flavonoids in the cocoon shell of *B. mori* differ from those in the leaves of *M. alba* L., based on analyses of paper chromatography (Fujimoto et al., 1959). Fujimoto and Hayashiya (1972) reported that the flavonoids in the cocoon shell of *B. mori* were also made from an artificial diet containing quercetin, rutin or isoquercitrin. Unfortunately, they did not report the chemical structures of the flavonoids in the cocoon shell. These flavonoid 5-glucosides in the cocoon shell may be produced by *B. mori* itself from the quercetin derivatives, contained in mulberry leaves.

The metabolism of quercetin and its glucosides have been investigated in mammals. Isoquercitrin is deglycosylated and then glucuronidated during passage across the epithelium of the rat small intestine (Gee et al., 2000). Quercetin is conjugated by UDP-glucuronosyltransferase from human liver cell-free extracts at four hydroxyl groups (C-3, C-7, C-3′ or C-4′), but not at the C-5 hydroxyl group (Day et al., 2000). Furthermore, it has been reported that sulfated and methylated quercetin are produced when quercetin is metabolized in rats (Crespy et al., 1999). Flavonoid glucuronides, sulfates and methyl flavonoids were not found in the cocoon shell of *B. mori*; hence, the processes of metabolic conversion of flavonoids by *B. mori* are thought to differ from that of mammals. *B. mori* may have an unusual glucosyltransferase that can transfer a glucose moiety to the C-5 hydroxyl group of quercetin.

Although the functions of the flavonoid 5-glucosides in the cocoon shell remain unclear, the binding of a glucose moiety to quercetin increases hydrophilicity and may enable *B. mori* to control some biological activity of quercetin.

3. Experimental

3.1. Experimental procedures

UV-vis spectra were recorded using a Jasco U-530 iRM spectrophotometer. A Shimadzu SPD-M10Avp photodiode array detector was connected to the HPLC system. UV-vis spectral shifts with shift reagents were measured according to Markham (1982). FT–IR spectra were measured by film state casting of samples onto the KRS-5 window, using a Jasco FT/IR-420. Relative molecular masses were determined by FD–MS using a double-focusing gas chromatograph mass spectrometer equipped with a field-desorption apparatus (M-2500, Hitachi) (Takaichi, 1993). ^{1}H and ^{13}C NMR spectra in DMSO-d_6 with TMS as an internal standard were recorded using either a Varian Unity ANOVA 600 spectrometer (600 MHz), a Varian Unity Plus 500 spectrometer (500 MHz) or a Varian Unity Plus 400 spectrometer (400 MHz).

3.2. Biological materials

Three races of *Bombyx mori* with different cocoon colors were used, namely, Multi-Bi of light green cocoons, Daizo (G) of pale yellow ones and BC 25 of white ones. BC25 is an improved race of Shi25gou. Three races are stocked in this institute. The larvae of each race were reared on the leaves of *Morus alba* L.

3.3. Isolation and purification

Pigments were extracted from the cocoon shell of Multi-Bi (300 g) by MeOH–H_2O (2:1, v/v) at 60 °C for 1 h. Crude extracts were filtered and evaporated to dryness, and were then dissolved in MeOH–H_2O (1:1, v/v) and loaded on a column of Sephadex LH-20 (1.0×50 cm, Amersham Pharmacia Biotech). Compound **2** was eluted with MeOH–H_2O (3:7, v/v), compound **1** with MeOH–H_2O (1:1, v/v) and compound **3** with MeOH–H_2O (3:1, v/v). Purification by Sephadex LH-20 column chromatography was repeated. Finally, each fraction was purified by a preparative HPLC column of Nova-Pack C18 (7.8×300 mm, Waters). Compounds **2**, **1** and **3** were eluted with H_2O–MeOH (87:13, v/v), H_2O–MeOH (81:19, v/v) and H_2O–MeOH (18:7, v/v), respectively, at a constant flow rate of 3.0 ml min^{-1}. HPLC equipped with a Nova-Pak C18 column (3.9×150 mm, Waters) was also used analytically. Water was the eluent, followed by a linear gradient of MeOH to 100% at 30 min at a constant flow rate of 2.0 ml min^{-1}.

3.4. Hydrolysis of flavonoid glucosides

Compound **1** was dissolved in H_2O and incubated with 0.005% β-glucosidase (E.C.3.2.1.21) from Almonds (Worthington Biochemical Corporation) for 24 h at 37 °C.

The flavonoid moiety was dissolved in MeOH to separate it from the sugar moiety. The sugar moiety was dissolved in H_2O and analyzed by silica gel 60 HPTLC (Merck) developed with Me_2CO–2 mM NaOAc (17:3, v/v). Sugar was detected by spraying with 0.2% naphthoresorcinol in Me_2CO–3N H_3PO_4 (5:1, v/v) and then heated at 105 °C for 5 min. D-Glucopyranose (Chemprosa Holding AG), D-galactose (Pfanstiehl) and D-mannose (Pfanstiehl) were used as standards.

3.5. Compound **1**

Quercetin 5,4′-di-*O*-β-D-glucopyranoside; 50 mg; UV λ_{max}^{MeOH}: 359, 251; +$AlCl_3$ 420, 262; +$AlCl_3$+HCl 419, 261; +NaOMe 409, 279; +NaOAc 378, 272; +NaOAc+H_3BO_3 359, 251 nm. ^{1}H NMR spectral data (600 MHz, DMSO-d_6): δ 7.70 (1H, *d*, *J* = 1.8 Hz, H-2′), δ7.61 (1H, *dd*, *J* = 1.8, 8.4, H-6′), δ 7.25 (1H, *d*, *J* = 8.4 Hz, H-5′), δ 6.77 (1H, *d*, *J* = 1.8 Hz, H-6), δ 6.67 (1H, *d*, *J* = 1.8 Hz, H-8), δ 4.84 (1H, *d*, *J* = 7.2 Hz, H-1 in 4′-*O*-glc), δ 4.80 (1H, *d*, *J* = 7.2 Hz, H-1 in 5-*O*-glc). ^{13}C NMR spectral data (150 MHz, DMSO-d_6) are shown in Table 1. Assignments were based on ^{1}H–^{1}H COSY, NOESY and HSQC correlations.

3.6. Compound **2**

Quercetin 5,7,4′-tri-*O*-β-D-glucopyranoside; 2 mg; UV λ_{max}^{MeOH}: 359, 251; +$AlCl_3$ 420, 262; +$AlCl_3$+HCl 420, 262; +NaOMe 414, 270; +NaOAc 359, 252; +NaOAc+H_3BO_3 359, 250 nm. ^{1}H NMR spectral data (500 MHz, DMSO-d_6): δ 7.75 (1H, *d*, *J* = 2.5 Hz, H-2′), δ 7.64 (1H, *dd*, *J* = 2.0, 8.5 Hz, H-6′), δ 7.26 (1H, *d*, *J* = 9.0 Hz, H-5′), δ 7.04 (1H, *d*, *J* = 2.5 Hz, H-8¶), δ 6.86 (1H, *d*, *J* = 2.0 Hz, H-6¶), δ 5.15 (1H, *d*, *J* = 6.5 Hz, H-1 in 7-*O*-glc), δ 4.91 (1H, *d*, *J* = 7.5 Hz, H-1 in 5-*O*-glc§), δ 4.83 (1H, *d*, *J* = 7.0 Hz, H-1 in 4′-*O*-glc§) (¶§Corresponding assignments may be reversed.) ^{13}C NMR spectral data (125 MHz, DMSO-d_6) are shown in Table 1.

3.7. Compound **3**

Quercetin 5-*O*-β-D-glucopyranoside; 5 mg; UV λ_{max}^{MeOH}: 367, 252; +$AlCl_3$ 453, 269; +$AlCl_3$+HCl 425, 263; +NaOMe 420 (dec), 333, 247; +NaOAc 386, 272; +NaOAc+H_3BO_3 381, 266 nm. ^{1}H NMR spectral data (400 MHz, DMSO-d_6) δ 7.65 (1H, *d*, *J* = 2.0 Hz, H-2′), δ 7.52 (1H, *dd*, *J* = 2.0, 8.4 Hz, H-6′), δ 6.88 (1H, *d*, *J* = 8.4 Hz, H-5′), δ 6.75 (1H, *d*, *J* = 2.4 Hz, H-6), δ 6.62 (1H, *d*, *J* = 2.0 Hz, H-8), δ 4.78 (1H, *d*, *J* = 7.2 Hz, H-1 in 5-*O*-glc). ^{13}C NMR spectral data (100 MHz, DMSO-d_6) are shown in Table 1.

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