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Regioselective formation of quercetin 5-*O*-glucoside from orally administered quercetin in the silkworm, *Bombyx mori*

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Abstract

The cocoons of some races of the silkworm, *Bombyx mori*, have been shown to contain 5-*O*-glucosylated flavonoids, which do not occur naturally in the leaves of their host plant, mulberry (*Morus alba*). Thus, dietary flavonoids could be biotransformed in this insect. In this study, we found that after feeding silkworms a diet rich in the flavonol quercetin, quercetin 5-*O*-glucoside was the predominant metabolite in the midgut tissue, while quercetin 5,4′-di-*O*-glucoside was the major constituent in the hemolymph and silk glands. UDP-glucosyltransferase (UGT) in the midgut could transfer glucose to each of the hydroxyl groups of quercetin, with a preference for formation of 5-*O*-glucoside, while quercetin 5,4′-di-*O*-glucoside was predominantly produced if the enzyme extracts of either the fat body or silk glands were incubated with quercetin 5-*O*-glucoside and UDP-glucose. These results suggest that dietary quercetin was glucosylated at the 5-*O* position in the midgut as the first-pass metabolite of quercetin after oral absorption, then glucosylated at the 4′-*O* position in the fat body or silk glands. The 5-*O*-glucosylated flavonoids retained biological activity in the insect, since the total free radical scavenging capacity of several tissues increased after oral administration of quercetin.

Keywords: Silkworm; *Bombyx mori*; Mulberry; Morus alba; Moraceae; Flavonoid; Flavonol; Quercetin; UDP-glucosyl transferase; Glucosylation; Antioxidant; Regioselectivity

1. Introduction

Uptake and utilization of dietary flavonoids is widespread in insects, in particular in the Lepidoptera. It has been reported that some insects sequester plant flavonoids into their body cuticles for protection against natural enemies, or into their wings to increase attractiveness to mates (Simmonds, 2003). Larvae of the silkworm *Bombyx mori* sequester flavonoids into their cocoons from the leaves of their host plant, the mulberry tree (*Morus alba*) (Fujimoto et al., 1959). Recently, Tamura et al. (2002) identified three flavonol glucosides, quercetin-5-*O*-glucoside (**5**), quercetin 5,4′-di-*O*-glucoside (**2**), and quercetin 5,7,4′-tri-*O*-glucoside from the cocoon shell. However, these compounds were not present in mulberry leaves, in which flavonol glycosides with a sugar group at the 3-*O* position in the C ring such as isoquercitrin (quercetin 3-*O*-glucoside), rutin (quercetin 3-*O*-rutinoside), quercetin 3-*O*-(6-malonylglucoside), and astragalin (kaempferol 3-*O*-glucoside) are naturally occurring (Doi et al., 2001; Katsube et al., 2006; Onogi et al., 1994). Thus, we can infer that flavonoids absorbed from their diet are modified in the insect for using these compounds to increase fitness. In insects, the formation of glucoside is the predominant pathway for dietary flavonoids (Hopkins and Ahmad, 1991; Lahtinen et al., 2006; Salminen et al., 2004; Wiesen et al., 1994), and the glucosylation of polyphenolics in insects is catalyzed by UDP-glucosyltransferase (UGT) (Ahmad and Hopkins, 1993; Rausell et al., 1997; Real et al., 1991), suggesting the possibility

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that a UGT enzyme that can transfer a glucose moiety to the C-5 position of the flavonols is functioning in *B. mori*.

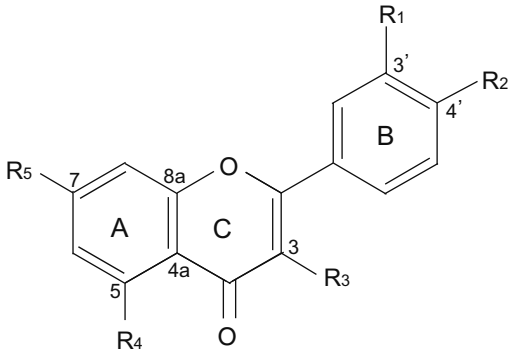
In the present study, we identified flavonoids distributed in the tissues of the silkworms fed a diet supplemented with flavonol quercetin (**10**), and showed that the flavonoids helped increase the anti-oxidative state of the tissues. Further, we determined the in vitro activity of UGT in transferring glucose to each OH group of quercetin (**10**) in the midgut, fat body, and silk glands, in order to establish the metabolic pathway of dietary quercetin (**10**) in the insect. This is the first study on the metabolism of flavonoid through the regiospecific glycosylation pathway in insects and we demonstrate the first example of glycosylation of quercetin (**10**) by a UGT enzyme with the preferred 5-*O* regioselectivity.

2. Results and discussion

2.1. Structural elucidation of cocoon shell flavonoids

Tamura et al. (2002) demonstrated that the cocoon shell of the silkworm (the race "Multi-Bi") contained quercetin 5-*O*-glucoside (**5**), quercetin 5,4′-di-*O*-glucoside (**2**), and quercetin 5,7,4′-tri-*O*-glucoside. In the present study, we isolated 9 flavonoids (compounds **1**–**6**, **10**–**12**) from the cocoon shell of the race "Pure-Mysore" reared on mulberry leaves. LC–MS analysis of the isolated flavonoids showed deprotonated ions $[M-H]^-$ with peaks at *m/z* 625 (compounds **1**–**4**), 609 (**11**), 463 (**5** and **6**), 447 (**12**), and 301 (**10**), respectively. In addition, reaction with β-glucosidase gave quercetin (**10**) or kaempferol, suggesting that compounds **1**–**4** were quercetin diglucosides, **5** and **6** were quercetin monoglucosides, **11** was kaempferol diglucoside, and **12** was kaempferol monoglucoside.

To identify the glycosylation sites of the flavonoids, the UV–Vis spectra were analyzed in the presence of the shift reagents as reported by Day et al. (2000). Obtained data identified quercetin 3,7-di-*O*-glucoside (**1**) quercetin 5,4′-di-*O*-glucoside (**2**), quercetin 3,3′-di-*O*-glucoside (**3**), quercetin 5,3′-di-*O*-glucoside (**4**), quercetin 5-*O*-glucoside (**5**), quercetin 7-*O*-glucoside (**6**), quercetin (**10**), kaempferol 5,4′-di-*O*-glucoside (**11**), and kaempferol 5-*O*-glucoside (**12**) (Table 1, Fig. 1). Among these flavonoids, quercetin 5,3′-di-*O*-glucoside (**4**) and kaempferol 5,4′-di-*O*-glucoside (**11**) are novel natural products. To confirm the chemical structures of these novel compounds, their structures were characterized by ^{1}H NMR, ^{13}C NMR, and high-resolution ESI mass spectroscopic analyses. The negative HR-FTICR-MS spectrum of **4** showed a prominent peak at *m/z* 625 $[M-H]^-$ (observed 625.1424, calculated 625.1410 for $C_{27}H_{29}O_{17}$); this value corresponds to a diglucoside



1 $R_1 = R_2 = R_4 = OH$, $R_3 = R_5 = O$-Glucose
2 $R_1 = R_3 = R_5 = OH$, $R_2 = R_4 = O$-Glucose
3 $R_2 = R_4 = R_5 = OH$, $R_1 = R_3 = O$-Glucose
4 $R_2 = R_3 = R_5 = OH$, $R_1 = R_4 = O$-Glucose
5 $R_1 = R_2 = R_3 = R_5 = OH$, $R_4 = O$-Glucose
6 $R_1 = R_2 = R_3 = R_4 = OH$, $R_5 = O$-Glucose
7 $R_1 = R_2 = R_4 = R_5 = OH$, $R_3 = O$-Glucose
8 $R_1 = R_3 = R_4 = R_5 = OH$, $R_2 = O$-Glucose
9 $R_2 = R_3 = R_4 = R_5 = OH$, $R_1 = O$-Glucose
10 $R_1 = R_2 = R_3 = R_4 = R_5 = OH$
11 $R_1 = H$, $R_3 = R_5 = OH$, $R_2 = R_4 = O$-Glucose
12 $R_1 = H$, $R_2 = R_3 = R_5 = OH$, $R_4 = O$-Glucose

Fig. 1. Structure of flavonoids either isolated or detected from the silkworm, *Bombyx mori*.

Table 1
Properties of flavonoids isolated from the cocoon shell of the silkworm, *Bombyx mori* (Race: Pure Mysore)

Cocoon flavonoid	Peak wavelength (nm)[a]					Aglycone	$[M-H]^-$[b] (*m/z*)
	MeOH	+NaOMe	+$AlCl_3$	+$AlCl_3$/HCl	+NaOAc		
1 Quercetin 3,7-di-*O*-glucoside	257, 358	266, 402	276, 436	272, 406	263, 415	Q[c]	625
2 Quercetin 5,4′-di-*O*-glucoside	251, 360	269, 317, 394	263, 420	263, 421	273, 382	Q	625
3 Quercetin 3,3′-di-*O*-glucoside	254, 365	272, 431	265, 423	265, 425	262, 383	Q	625
4 Quercetin 5,3′-di-*O*-glucoside	250, 364	272, 324, 414	262, 425	262, 425	274, 394	Q	625
5 Quercetin 5-*O*-glucoside	253, 368	271, 323, 411	272, 451	264, 429	267, 392	Q	463
6 Quercetin 7-*O*-glucoside	256, 373	Decomposed	269, 441	266, 432	259, 384	Q	463
10 Quercetin	256, 372	Decomposed	272, 450	267, 432	273, 385		301
11 Kaempferol 5,4′-di-*O*-glucoside	259, 358	275, 397	266, 418	265, 419	272, 380	K[d]	609
12 Kaempferol 5-*O*-glucoside	259, 362	375, 323, 405	268, 424	267, 423	273, 388	K	447

[a] UV spectral data were recorded in methanol and after addition of various shift reagents NaOMe, $AlCl_3$, $AlCl_3$+HCl, NaOAc.
[b] Deprotonated ion.
[c] Quercetin.
[d] Kaempferol.

derivative of quercetin (**10**). The D-glucosyl moiety and the structure of the quercetin moiety were identified by ^{1}H NMR, and ^{13}C NMR spectroscopic analyses (see data below). Moreover, the cross peak from C-5 (δ 159.74) to 5-*O*-Glc-H-1 (δ 4.88) of glucose and the cross peak from C-3′ (δ 146.81) to 3′-*O*-Glc-H-1 (δ 4.87) of glucose in the HMBC spectrum indicated that a glucose moiety was attached to the 5-*O* position and another to the 3′-*O* position of the quercetin (**10**) (data not shown).

The structure of **11** was also characterized by the same method used in the analysis of **4**. The negative HR-FTICR-MS spectrum of **11** showed a prominent peak at *m/z* 609 $[M-H]^-$ (observed 609.1470, calculated 609.1461 for $C_{27}H_{29}O_{16}$), indicating that **11** was a diglucosyl conjugate of kaempferol. The HMBC analysis confirmed that two glucopyranosyl moieties were conjugated to the 5-*O* and 4′-*O* positions of kaempferol. From these results, we concluded that **4** was quercetin 5,3′-di-*O*-β-D-glucopyranoside, and **11** was kaempferol 5,4′-di-*O*- β-D-glucopyranoside.

*2.2. Identification and quantification of flavonoid metabolites in the silkworm tissues after feeding with a quercetin (**10**) diet*

Using the flavonoids commercially available and authentic compounds purified from the cocoon shell, flavonoid metabolites in the tissues was analyzed by HPLC. LC-MS were also used to confirm that the metabolites were quercetin (**10**) or kaempferol glucosides based on the detected deprotonated ions $[M-H]^-$ at *m/z* 463, 625, 447, 609. Furthermore, decrease of the metabolite peaks with concomitant formation of either quercetin (**10**) or kaempferol was not observed when tissue samples were incubated with glucuronidase and sulfatase, suggesting that no conjugates other than glucosides existed in the silkworm tissues (data not shown). In the midgut tissues, quercetin 5,4′-di-*O*-glucoside (**2**), quercetin 5,3′-di-*O*-glucoside (**4**), quercetin 5-*O*-glucoside (**5**), quercetin 3-*O*-glucoside (**7**), and quercetin (**10**) were detected (Fig. 2A). Total flavonoid content in the midgut was 168 nmol/g fresh tissue, and 1 g fresh midgut tissue contained 124 nmol quercetin 5-*O*-glucoside (**5**) as the main metabolite (Table 2).

In the hemolymph, quercetin 3,7-di-*O*-glucoside (**1**), quercetin 5,4′-di-*O*-glucoside (**2**), quercetin, 3′-di-*O*-glucoside (**3**), quercetin 5,3′-di-*O*-glucoside (**4**), and quercetin 5-*O*-glucoside (**5**) were detected, with the most abundant metabolite being quercetin 5,4′-di-*O*-glucoside (**2**) (Fig. 2B). Whole silk glands showed a very similar flavonoid profile to that of hemolymph, with ca. 90% of the total flavonoids in the tissue being quercetin 5,4′-di-*O*-glucoside (**2**) (Fig. 2C, Table 2). These results suggest a second stage of biotransformation in the internal tissues with subsequent glucosylation at the 4′-*O* position of quercetin 5-*O*-glucoside (**5**), which is produced in the midgut as the first-pass metabolite of quercetin (**10**) after oral absorption.

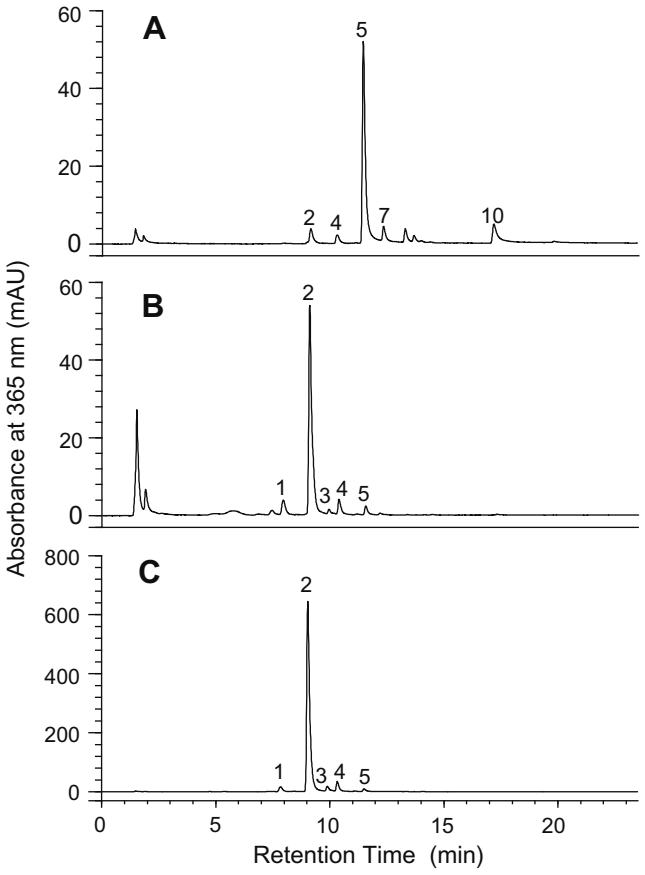


Fig. 2. Representative HPLC chromatograms of tissues obtained from the silkworm fed a quercetin-supplemented diet. A; midgut; B; hemolymph; C; silk glands. Detection was performed at 365 nm: **1**, quercetin 3,7-di-*O*-glucoside; **2**, quercetin 5,4′-di-*O*-glucoside; **3**, quercetin 3,3′-di-*O*-glucoside; **4**, quercetin 5,3′-di-*O*-glucoside; **5**, quercetin 5-*O*-glucoside; **7**, quercetin 3-*O*-glucoside; **10**, quercetin.

Table 2
Quantification of quercetin metabolites in the silkworm reared on a quercetin (**10**) supplemented diet[a]

Quercetin metabolite	Midgut (nmol/g fresh tissue)	Silk glands (nmol/g fresh tissue)	Hemolymph nmol/ml
1 Quercetin 3,7-di-*O*-glucoside	–	57.9 ± 6.4	7.5 ± 2.2
2 Quercetin 5,4′-di-*O*-glucoside	11.0 ± 1.8	1829 ± 277	59.3 ± 12.9
3 Quercetin 3,3′-di-*O*-glucoside	–	42.2 ± 4.1	1.0 ± 0.8
4 Quercetin 5,3′-di-*O*-glucoside	5.9 ± 1.8	82.1 ± 8.8	4.5 ± 1.2
5 Quercetin 5-*O*-glucoside	124.4 ± 63.8	27.5 ± 16.7	3.0 ± 0.8
7 Quercetin 3-*O*-glucoside	9.8 ± 1.6	–	–
10 Quercetin	16.8 ± 5.8	–	–
Total	168.0 ± 74.8	2039 ± 308	75.3 ± 17.8

[a] Values are the means ± SD ($n = 5$). Metabolite quantity is expressed as quercetin 5-*O*-glucoside (**5**) equivalents.

Kaempferol glycosides were not detected in the tissues examined after feeding with a quercetin diet. In addition, the cocoon shells of the silkworms reared with mulberry leaves had much lower amounts of kaempferol glycosides than quercetin glycosides, probably because the mulberry leaves contained smaller amounts of the former. However, we confirmed that kaempferol 5,4′-di-*O*-glucoside (**11**) and kaempferol 5-*O*-glucoside (**12**) were the main metabolites when the insects were fed a diet containing kaempferol only (data not shown).

2.3. Antioxidant capacity in the silkworm tissues

The tissue antioxidative status of silkworms fed a quercetin-supplemented diet was compared with that of insects fed a control diet (non-quercetin diet) (Table 3). The midgut extract of the quercetin-supplemented insects exhibited a strong antioxidant property; 1 g of fresh midgut contained an antioxidant equivalent to 3 μmol Trolox, about twice that of the control-diet silkworms. In addition, the larvae fed a quercetin diet also had stronger antioxidant activity in the silk glands than those fed a control diet. No significant difference between the groups was found in the antioxidative capacity of the hemolymph. It has been reported that the position of flavonoid glycosylation significantly affects their biological activity and potential benefits to humans (Day et al., 2000; Teixeira et al., 2005). For example, quercetin glucuronidated at the 3-*O* position has a protective effect against cardiovascular diseases (Yoshizumi et al., 2002), and has an anti-oxidant effect on copper-ion-induced lipid peroxidation in human plasma low-density lipoprotein (Moon et al., 2001). In contrast, substitution at the 3′-*O* and 4′-*O* positions results in a marked decrease in antioxidant activity (Yamamoto et al., 1999). Our study demonstrated that quercetin (**10**) exerted its anti-oxidant activity, at least in part, even after it was glucosylated at the 5-*O* position. However, total anti-oxidant activity in the hemolymph did not change, probably because the hemolymph mainly contained metabolites, such as quercetin 5,4′-di-*O*-glucoside (**2**), whose catechol moiety in the B-ring was glucosylated.

Table 3
Total antioxidant activity in tissues of the silkworms fed an experimental diet[a]

Diet group	TEAC (μmol/g fresh weight)[b]		Hemolymph TEAC (μmol/ml)[c]
	Midgut	Silk glands	
Control	1.84 ± 0.27	2.32 ± 0.36	2.60 ± 0.26
Quercetin (**10**) 0.5%	3.45 ± 0.60	6.73 ± 0.66	2.69 ± 0.28

[a] Values are means ± SD ($n = 5$).
[b] TEAC, Trolox-equivalent capacity. Data are expressed as μmol of Trolox equivalent/g fresh weight.
[c] Data are expressed as μmol of Trolox equivalent/ml.

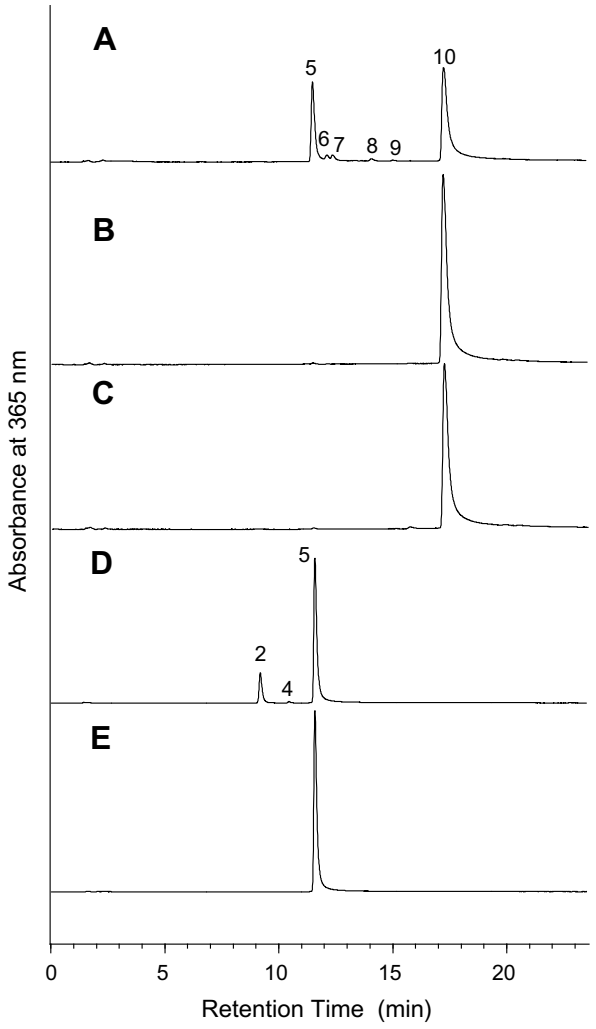


Fig. 3. HPLC chromatograms of silkworm UDP-glucosyltransferase assays: A, incubation mixture of midgut membrane fraction with quercetin (**10**) and UDP-glucose; B, incubation mixture of midgut membrane fraction with quercetin (**10**) without UDP-glucose; C, incubation mixture of midgut soluble fraction with quercetin (**10**) and UDP-glucose; D, incubation mixture of middle silk gland membrane fraction with quercetin-5-glucoside (**5**) and UDP-glucose; E, incubation mixture of middle silk glands membrane fraction with quercetin-5-glucoside (**5**) without UDP-glucose. Detection was performed at 365 nm: **2**, quercetin 5,4′-di-*O*-glucoside; **4**, quercetin 5,3′-di-*O*-glucoside; **5**, quercetin 5-*O*-glucoside; **6**, quercetin 7-*O*-glucoside; **7**, quercetin 3-*O*-glucoside; **8**, quercetin 4′-*O*-glucoside; **9**, quercetin 3′-*O*-glucoside; **10**, quercetin.

2.4. Determination and tissue distribution of UDP-glucosyl transferase (UGT) activity

Fig. 3A–C indicate typical HPLC chromatograms of quercetin (**10**) incubation with the midgut extracts. When the midgut membrane extract was incubated with UDPG and quercetin, five metabolites (**5**–**9**) were formed (Fig. 3A). Incubation of the sample with β-glucosidase resulted in the disappearance of the metabolite peaks, with concomitant formation of quercetin (**10**), indicating that these metabolites were glucoside derivatives of quercetin (**10**) (data not shown). These metabolites were identified

based on their co-elution with authentic reference standards (**5**: quercetin 5-*O*-glucoside, **6**: quercetin 7-*O*-glucoside, **7**: quercetin 3-*O*-glucoside, **8**: quercetin 4′-*O*-glucoside, **9**: quercetin 3′-*O*-glucoside) (Fig. 3A). However, no glucosylation of quercetin (**10**) occurred in the absence of UDP-glucose (Fig. 3B), nor did glucosides form when the cytosolic extract was used instead of the membrane fraction (Fig. 3C). The membrane fractions from other tissues also had UDP-glucose-dependent activity that glucosylated the OH groups of quercetin (**10**), while the cytosolic fractions had no activity (data not shown). The results of the present study thus agree with the report by Ahmad and Hopkins (1992) that UGTs were always concentrated in the heavy cellular particles, probably associated with microsomes. UDP-glycosytransferases are a large family of enzymes capable of transferring the glycosyl group from the substrate donor (UDP-glucose, UDP-glucuronic acid, UDP-galactose, etc.) to wide ranges of substrate acceptor molecules (Bowles et al., 2005; Bowles et al., 2006; Coutinho et al., 2003). In general, glucosylation seems to be a more important pathway for the detoxication of plant phenolics in insects (Ahmad and Hopkins, 1993; Rausell et al., 1997; Real et al., 1991), rather than glucuronidation, which is the predominant pathway in mammals. We confirmed that the silkworm UGTs use only UDP-glucose as the substrate donor (data not shown).

UGT activity toward quercetin (**10**) in each tissue was calculated, and the values, shown in Table 4, indicate that the rate of glucosylation of OH positions of quercetin varied between tissues. UGT in the midgut produced 5-, 3-, 7-, 3′-, and 4′-*O*-glucoside, with a preference for the formation of the 5-glucoside. The fat body had strong activity of UGT that glucosylated quercetin at the 3-OH and 4′-OH groups. UGT in both the posterior and middle silk glands showed a regiospecificity toward the glucosylation at the 7-*O* position of quercetin (**10**). Subsequently, the capacity of the silkworm membrane extract of each tissue to transfer glucose from UDP-glucose to quercetin-5-*O*-glucoside (**5**) was investigated. Fig. 3D shows that two metabolites (**2**: quercetin 5,4′-di-*O*-glucoside and **4**: quercetin 5,3′-di-*O*-glucoside) were formed when the membrane extract of the middle silk glands was incubated with UDP-glucose and quercetin 5-*O*-glucoside (**5**). However, no glucosylation of quercetin 5-*O*-glucoside (**5**) occurred in the absence of UDP-glucose (Fig. 3E). The UGT activity toward quercetin 5-*O*-glucoside (**5**) in each tissue is demonstrated in Table 5. The rate of glucosylation for quercetin 5-*O*-glucoside (**5**) was very low with the midgut membrane extract, although the activity of glucosylation toward quercetin aglycone was high (see Table 4). The rate of glucosylation toward quercetin 5-*O*-glucoside (**5**) was very high in the fat body and in both the posterior and middle silk glands, in which the dominant product, quercetin 5,4′-di-*O*-glucoside (**2**), was produced along with a minor product, quercetin 5,3′-di-*O*-glucoside (**4**). Interestingly, no products were formed other than quercetin-5,4′-diglucoside (**2**) and quercetin-5,3′-diglucoside (**4**), irrespective of the tissues investigated. Thus, the in vitro study indicates that glucosylation of quercetin (**10**) at the 5-*O* position occurs preferably in the intestinal tissue followed by subsequent glucosylation at the 4′-*O* and 3′-*O* positions in the fat body and silk gland tissues.

Recently, there has been increasing interest in the biocatalytic synthesis of glycosides with potential medicinal properties using regioselective UGTs (Kramer et al., 2003; Lim, 2005; Weis et al., 2006; Willits et al., 2004), because the chemical synthesis of specific glycosides is quite complicated. For example, to produce any single monoglucoside of quercetin (**10**), four other hydroxyl groups should be protected in each step of the reactions, but this may reduce the yield of the target product. The regioselectivity of UGTs can offer a potential solution to overcome the problems of chemical synthesis of specific glycosides. In order to produce specific quercetin glucosides, a large multigene family of UGTs in *Arabidopsis thaliana* has been expressed as recombinant enzymes in *Escherichia coli* by Lim et al. (2004). They analyzed the activity of 91 recombinant enzymes for in vitro activity toward quercetin (**10**) and found that 29 enzymes were capable of glucosylating the compound, and that different monoglucosides of quercetin (**10**) (3-, 7-, 3′-, and 4′-*O*-glucosides) were synthesized by using these UGTs. However, significant activity toward 5-*O* position of flavonols has not been observed by any of the enzymes assayed. Similarly, studies of the regioselectivity of glucuronidation of quercetin (**10**) demonstrated that neither human liver and intestinal cell free extracts nor individual UGT isoforms generate 5-glucuronosyl metabolites of quercetin (**10**) (Boersma et al., 2002; Day et al., 2000). The OH group at the C-5 position of flavone is

Table 4
Glucosyltransferase activity toward quercetin (**10**) in the silkworm tissues[a]

Tissue	Specific activity (pkat/mg protein)				
	3-*O*-Glc	5-*O*-Glc	7-*O*-Glc	3′-*O*-Glc	4′-*O*-Glc
Midgut	3.0 ± 1.0	38.7 ± 4.7	3.2 ± 0.8	0.5 ± 0.2	1.3 ± 0.5
Fat body	61.4 ± 20.2	18.0 ± 3.8	18.0 ± 3.8	5.3 ± 0.8	63.5 ± 12.7
Posterior silk glands	–	–	133 ± 21	0.5 ± 0.3	8.7 ± 1.5
Middle silk glands	–	–	186 ± 27	–	10.8 ± 2.2

[a] Values are means ± SD ($n = 5$).

Table 5
Glucosyltransferase activity toward quercetin 5-*O*-glucoside (**5**) in the silkworm tissues[a]

Tissue	Specific activity (pkat/mg protein)			
	3-*O*-Glc	7-*O*-Glc	3′-*O*-Glc	4′-*O*-Glc
Midgut	–	–	0.5 ± 0.2	4.0 ± 1.7
Fat body	–	–	8.8 ± 2.3	47.0 ± 8.2
Posterior silk glands	–	–	3.7 ± 1.0	109 ± 22
Middle silk glands	–	–	4.7 ± 1.3	169 ± 26

[a] Values are means ± SD ($n = 5$).

the most inert site for glucuronidation, since the carbonyl group at C-4 could produce steric hindrance for the adjacent OH-group at C-5. In addition, strong intra-molecular hydrogen bonding of OH at C-5 with the carbonyl group at C-4 also prevents glycosylation of OH at C-5 (Zhang et al., 2006). Therefore, the inert glycosylation activity of OH-group at C-5 could explain why 5-*O*-glycosylated flavones and flavonols are very rare compounds as reported by Iwashina et al. (1995). We consider that the unique silkworm midgut UGT with a regiospecificity toward the glycosylation of the 5-*O* position of quercetin (**10**) would be useful as a novel biocatalyst for the production of potential pharmaceuticals.

3. Conclusions

In conclusion, our results demonstrated for the first time that a UGT with a preferred 5-*O* regioselectivity for quercetin (**10**) glycosylation was operative in the silkworm; however, the physiological function of the "unusual" regioselectivity of the enzyme remains to be established. Besides the antioxidant potential, 5-*O*-glucosylated flavonoids might act as antibiotic and antiviral agent, or for protection against predators. Further research to obtain more information about the role of 5-*O*-glucosylated flavonoids in the insect and the isolation of the UGT enzyme are necessary to understand the functional significance of the unique glucosylation pathway.

4. Experimental

4.1. Chemicals

Quercetin (**10**), uridine-5′-diphospho (UDP)-glucose, rutin, and β-glucosidase from almond were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Quercetin 3-*O*-glucoside (**7**) and quercetin 4′-*O*-glucoside (**8**), kaempferol 3-*O*-glucoside, and kaempferol were purchased from Extrasynthase (Genay, France). Trolox (6-hydroxy-2, 5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble analogue of vitamin E, was from EMD Biosciences (La Jolla, CA, USA). 2,2′-Azinobis- (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was obtained from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals and solvents used were of either analytical or HPLC grade.

4.2. Isolation of flavonoids from the cocoon shell of the silkworm

To obtain authentic flavonoids available for the identification and quantification of quercetin metabolites in the silkworm tissues, flavonoids were isolated from the cocoon shell of the silkworm race "Pure-Mysore". These insects were reared on mulberry leaves (*M. alba*) throughout the larval stage. Flavonoids were extracted and purified from the pale yellow cocoon shell as described in a previous report (Tamura et al., 2002) with some modifications. Briefly, MeOH–H_2O (7:3, v/v) extract from the cocoon shell was concentrated by evaporation and diluted with distilled H_2O. This aqueous solution was applied to a solid phase extraction cartridge (Oasis HLB, 35 ml, Waters, Milford, MA, USA). After washing with distilled H_2O, the column was eluted with MeOH–H_2O (1:1, v/v) followed by MeOH. The MeOH–H_2O (1:1, v/v) fraction contained mainly flavonoid diglucosides, and the MeOH fraction contained monoglucosides and aglycones. Each fraction was concentrated by evaporation and loaded on a 2.6 × 60 cm Toyopearl HW-40F column (TOSOH, Tokyo, Japan) eluted with a linear gradient of MeOH:H_2O:H-CO_2H (40:59.9:0.1, v/v/v) to MeOH:HCO_2H (99.9:0.1,v/v). Fractions containing flavonoids were further purified by preparative reversed-phase HPLC (Nova-Pak C18, 19 × 300 mm, Waters).

4.3. Deconjugation of isolated flavonoids

Hydrolysis with β-glucosidase was carried out to identify the flavonoids isolated from the cocoon shell. Each flavonoid (ca 2.5 μg) was treated with 1.3 U β-glucosidase in 1 ml 25 mM acetate buffer (pH 5.0) at 37 °C for 1 h. After incubation, four volumes of MeOH were added to stop the enzyme reaction. The aglycones formed were identified by HPLC using commercially available standards.

4.4. Identification of conjugation positions by UV spectra

Information on the conjugation positions of flavonoids was determined indirectly by UV spectra according to Markham (1982). Standards of flavonols commercially available (quercetin (**10**), quercetin 3-*O*-glucoside (**7**), quercetin 4′-*O*-glucoside (**8**), kaempferol, kaempferol 3-*O*-glucoside), and each isolated flavonoid glucoside were dissolved in MeOH (20 ml) and diluted until the absorbance of the maxima was between 0.6 and 0.7. Shift reagents were added sequentially to the freshly prepared stock solution (2.5 ml), and the UV spectra were recorded on a UV-2500 PC spectrometer (Shimadzu, Tokyo, Japan).

4.5. Structural analysis for novel flavonol glucosides

High-resolution ESI mass analysis for novel glucosyl conjugates purified from the cocoon shell were carried out using an Apex II 70e Fourier-transform ion cyclotron resonance (FTICR) mass spectrometer (Bruker, Gothenburg, Sweden). These compounds were characterized by ^{1}H NMR and ^{13}C NMR spectroscopic analyses using either a Bruker AVANCE 500 spectrometer (500 MHz for ^{1}H; 125 MHz for ^{13}C) or a Bruker AVANCE 800 spectrometer (800 MHz for ^{1}H; 200 MHz for ^{13}C) in CD_3OD. Chemical shifts were expressed in ppm relative to tetramethylsilane (TMS) as an internal standard.

4.6. Chemical synthesis of quercetin 3′-glucoside

Among quercetin monoglucosides, quercetin 3′-*O*-glucoside (**9**) was neither commercially available nor abundant in the cocoon shell of the silkworm. To use quercetin 3′-*O*-glucoside (**9**) as an authentic standard, we synthesized it from quercetin 5,3′-di-*O*-glucoside (**4**) purified from the cocoon shell. Quercetin 5,3′-di-*O*-glucoside (**4**) (1 mg) was dissolved in MeOH–H_2O (20 ml, 7:3, v/v), and 4 ml 6 M HCl was added to the solution. Then the acidic solution was heated at 100 °C for 5 min. Acid hydrolysis at the 5-*O* position of quercetin (**10**) occurred much more rapidly than that at 3′-*O*, predominantly giving quercetin 3′-*O*-glucoside (**9**). The hydrolysate was diluted with distilled H_2O and applied to a solid phase extraction cartridge (Oasis HLB, 20 ml, Waters). After washing with distilled H_2O, the column was eluted with MeOH. The eluent was concentrated by evaporation and loaded to a preparative reversed-phase HPLC (Nova-Pak C18, 300 × 19 mm i.d., Waters) to yield quercetin 3′-*O*-glucoside (**9**). The glucosylation site of the obtained compound was confirmed by the pattern of UV spectra produced by the shift reagents as described above.

4.7. Dietary administration of quercetin (*10*) and collection of silkworm tissues

The larvae of Pure-Mysore were reared on a diet of mulberry leaf powder from hatching to the 4th ecdysis (Hirayama et al., 1997). Female larvae just after the 4th ecdysis were randomly divided into two groups. The control group received a semi-synthetic diet for 5 days. The semi-synthetic diet had a composition similar to that of the diet used during the young larval stage, except that the mulberry leaf powder was omitted and the contents of soybean protein and cellulose powder were increased to 40 g/100 g and 15.3 g/100 g, respectively. The test group received the semi-purified diet supplemented with 0.5 g quercetin (**10**) per 100 g for 5 days. After the larvae fed on the respective diets *ad libitum* for 5 days, the hemolymph was collected from the larvae by cutting prolegs. Two volumes (v/v) of MeOH were added to the collected hemolymph, and denatured protein was removed by centrifugation at 20,000*g* for 10 min, then immediately frozen at −80 °C. After the hemolymph was drawn, the larvae were quickly dissected and the silk glands and midgut were collected. These tissues were well rinsed with an ice-cold 0.85% KCl solution, then rapidly frozen and stored at −80 °C until analysis.

4.8. Preparation of tissue samples for flavonoid analyses and antioxidant activity

The MeOH extracts of hemolymph stored at −80 °C were thawed at room temperature and centrifuged at 20,000*g* for 10 min, after which the precipitates were removed. The supernatant was passed through a 0.45 μm filter and used for analysis.

Frozen midgut and whole silk glands were homogenized in three volumes (v/w) of MeOH–H_2O (7:3, v/v) at 4 °C. After centrifugation at 20,000*g* for 10 min, the precipitates were re-homogenized in MeOH–H_2O (7:3, v/v). After centrifugation, the second extract was combined with the first supernatant. This step was performed again. The combined extract was diluted with MeOH to a constant volume and filtered, and then the supernatant was used for HPLC analysis and for determination of the total antioxidant activity.

4.9. HPLC analysis

Flavonoids in the tissues were identified and quantified with a Shimadzu HPLC system equipped with an LC-7A pump, a CTO-10A column oven, and an SPD-7AV UV–Vis detector monitoring at 365 nm. Samples were loaded onto a Nova-Pak C18 column (150 × 3.9 mm i.d.). Elution was performed using H_2O:HCO_2H (99.8:0.2 v/v) as solvent A and CH_3CN as solvent B at a flow rate of 1.0 ml/min at 40 °C. Flavonoids were separated out with linear gradient from 7% B to 40% B for 20 min and then to 100% for 10 min. Quercetin (**10**) metabolites were identified based on their co-elution with either commercially available standards or authentic reference samples purified from the cocoon shell. Flavonoids were quantified using quercetin 5-*O*-glucoside (**5**) as a reference compound.

4.10. LC–MS analysis

LC–MS analyses of flavonoids in the cocoon shells and tissues were carried out by an HP 1100 series HPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with an HP 1100MSD mass spectrometer. An aliquot (10 μl) of a sample was injected into the LC–MS and separated by a C18 reversed phase column, 150 mm × 2.0 mm i.d. (Nova-Pak C18, Waters), at a flow rate of 0.3 ml/min. The column temperature was maintained at 40 °C. The mobile phase consisted of solvents A (0.2% aq. HCO_2H) and B (0.2% HCO_2H in CH_3CN). Metabolites were separated with a linear gradient from 7% B to 40% B over 20 min and then to 100% for 5 min. UV detection was carried out using an HP 1100 photodiode array detector to facilitate peak assignment. UV–Vis spectra were recorded in the 190–450 nm range, and the chromatograms were acquired at 190, 365, and 420 nm. The eluent was ionized by negative electrospray ionization. The electrospray mass spectrometer conditions were as follows: negative ion mode; fragmentor voltage, 70 V; capillary voltage, 3500 V; drying gas (nitrogen) flow, 10.0 L/min; nebulizer pressure, 25 psig; drying gas temperature, 350 °C. The flavonoid conjugates were identified on the basis of their retention times, UV–Vis spectra, and electrospray mass spectra.

4.11. In vitro assay of UDP-glucosyltransferase (UGT)

Female larvae after the 4th ecdysis were fed semi-synthetic diet for 5 days, after which the larvae were dissected

and the midgut, posterior and middle silk glands, and fat body were collected. These tissues were well rinsed with an ice-cold 0.85% KCl solution, then rapidly frozen and stored at −80 °C until analysis.

Frozen organs or tissues were homogenized in four volumes (v/w) of 0.1 M potassium phosphate buffer (pH 7.0) and centrifuged at 23,000*g* for 20 min at 4 °C. The supernatants were saved for measuring the fractional distribution of UGT. The precipitates were re-homogenized in 0.1 M potassium phosphate buffer (pH 7.0) containing 0.8% sodium cholate at 4 °C. After extraction with shaking for 30 min at 4 °C, the homogenates were centrifuged at 23,000*g* for 20 min. The supernatant was used as the enzyme solution of the membrane fraction.

UGT was assayed as described by Ahmad and Hopkins (1993). Briefly, the incubation mixtures consisted of 0.1 M potassium phosphate (pH 7.0), 5 mM UDPG, 25 mM $MgCl_2$, 5 mM D-glucuronolactone (as an inhibitor of β-glucosidase), 0.1 mM quercetin (**10**) or quercetin 5-*O*-glucoside (**5**), and enzyme preparation in a final incubation volume of 0.1 ml. The enzyme reactions were started by addition of UDP-glucose, then incubated for 30 min at 37 °C. The reaction was stopped by the addition of MeOH (0.2 ml). Precipitated protein was removed by centrifugation at 20,000*g* for 10 min, then the supernatant was used for determination of glucosides by HPLC. Control incubations were run, in which the enzyme source, substrates, or UDP-glucose was omitted. The glucosyl metabolites formed by tissue extracts were measured on the basis of the peak area in the HPLC chromatogram at 365 nm, using commercially available standards or authentic reference samples purified from the cocoon shell, as described above. The protein concentration was measured by a commercial assay kit (Coomasie Plus, Pierce, Rockford, IL, USA), using bovine serum albumin as a standard.

4.12. Quantification of antioxidant activity of the silkworm tissues

Aqueous methanolic extracts of hemolymph, midgut, and silk glands were tested for their ability to quench the ABTS radical as previously reported (Re et al., 1999). The ABTS stock solution (7.0 mM) was prepared and reacted with 2.45 mM potassium persulfate (final concentration) to generate the radical cation. After the mixture was kept in the dark at room temperature for 12–16 h to allow the completion of radical generation, the concentration of the ABTS radical solution was adjusted with MeOH to an absorbance of 0.7 ± 0.02 (mean ± SD) at 734 nm. To determine the scavenging activity, 1 ml of diluted ABTS solution was added to 10 μl sample solution, and the absorbance was measured at 734 nm 5 min after the initial mixing, using MeOH as blank. The percentage inhibition was calculated by the equation% inhibition $= (A_c - A_S)/A_c \times 100$ where A_c is the absorbance of the control and As is the absorbance of the samples. The Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) standard solution (final concentration of 0–0.5 mM) in EtOH–H_2O (4:1, v/v) was prepared and assayed under the same conditions. The results were expressed in terms of trolox equivalent antioxidant capacity.

4.13. Quercetin 5,3′-di-O-β-D-glucopyranoside (compound **4**)

^{1}H NMR (CD_3OD): δ 3.434−3.48 (3H, *m*, 5-*O*-Glc-H-4, 5-*O*-Glc-H-5, 3′-*O*-Glc-H-4), 3.49–3.51 (3H, *m*, 5-*O*-Glc-H-3, 3′-*O*-Glc-H-3, 3′-*O*-Glc-H-5), 3.55 (1H, *dd*, *J* = 7.8 and 9.3 Hz, 3′-*O*-Glc-H-2), 3.63 (1H, *dd*, *J* = 7.8 and 9.3 Hz, 5-*O*-Glc-H-2), 3.75 (1H, *dd*, *J* = 5.2 and 12.1 Hz, 5-*O*-Glc-H-6a), 3.79 (1H, *dd*, *J* = 5.3 and 12.1 Hz, 3′-*O*-Glc-H-6a), 3.94 (1H, *dd*, *J* = 2.0 and 12.1 Hz, 5-*O*-Glc-H-6b), 3.97 (1H, *dd*, *J* = 2.1 and 12.1 Hz, 3′-*O*-Glc-H-6b), 4.87 (1H, *d*, *J* = 7.8 Hz, 3′-*O*-Glc-H-1), 4.88 (1H, *d*, *J* = 7.8 Hz, 5-*O*-Glc-H-1), 6.72 (1H, *d*, *J* = 2.1 Hz, H-8), 6.790 (1H, *d*, *J* = 2.1 Hz, H-6), 6.97 (1H, *d*, *J* = 8.6 Hz, H-5′), 7.90 (1H, *dd*, *J* = 2.0 and 8.6 Hz, H-6′), 8.14 (1H, *d*, *J* = 2.0 Hz, H-2′).

^{13}C NMR (CD_3OD): δ 62.48 (3′-*O*-Glc-C-6), 62.51 (5-*O*-Glc-C-6), 71.22 (5-*O*-Glc-C-4), 71.28 (3′-*O*-Glc-C-4), 74.76 (5-*O*-Glc-C-2), 74.85 (3′-*O*-Glc-C-2), 77.34 (5-*O*-Glc-C-3), 77.59 (3′-*O*-Glc-C-3), 78.47 (3′-*O*-Glc-C-5), 78.59 (5-*O*-Glc-C-5), 98.89 (C-8), 104.35 (3′-*O*-Glc-C-1), 104.45 (C-6), 104.93 (5-*O*-Glc-C-1), 107.42 (C-4a), 117.18 (C-5′), 117.77 (C-2′), 124.23 (C-1′), 124.89 (C-6′), 138.91 (C-3), 145.05 (C-2), 146.81 (C-3′), 150.26 (C-4′), 159.51 (C-8a), 159.74 (C-5), 165.10 (C-7), 173.84 (C-4), HR-FTICR-MS *m*/*z* 625.1424 $[M-H]^-$ (calculated for $C_{27}H_{29}O_{17}$ 625.1410).

4.14. Kaempferol 5,4′-di-O-β-D-glucopyranoside (compound **11**)

^{1}H NMR (CD_3OD): δ 3.39−3.42 1H, *m*, 4′-*O*-Glc-H-4), 3.48–3.51 (3H, *m*, 4′-*O*-Glc-H-2, 4′-*O*-Glc-H-3, 4′-*O*-Glc-H-5), 3.4 (1H, *dd*, *J* = 8.3 and 9.8 Hz, 5-*O*-Glc-H-4), 3.48 (1H, *ddd*, *J* = 2.0, 5.2 and 9.8 Hz, 5-*O*-Glc-H-5), 3.50 (1H, *dd*, *J* = 8.3 and 9.8 Hz, 5-*O*-Glc-H-3), 3.63 (1H, *dd*, *J* = 7.8 and 9.3 Hz, 5-*O*-Glc-H-2), 3.71 (1H, *dd*, *J* = 5.8 and 12.2 Hz, 4′-*O*-Glc-H-6a), 3.76 (1H, *dd*, *J* = 5.2 and 12.1 Hz, 5-*O*-Glc-H-6a), 3.92 (1H, *dd*, *J* = 2.2 and 12.2 Hz, 4′-*O*-Glc-H-6b), 3.943 (1H, *dd*, *J* = 2.0 and 12.1 Hz, 5-*O*-Glc-H-6b), 4.87 (1H, *d*, *J* = 7.8 Hz, 5-*O*-Glc-H-1), 5.01–5.03 (1H, *m*, 4′-*O*-Glc-H-1), 6.66 (1H, *d*, *J* = 2.1 Hz, H-8), 6.791 (1H, *d*, *J* = 2.1 Hz, H-6), 7.22 (2H, *d*, *J* = 9.0 Hz, H-3′ and 5′), 8.18 (2H, *d*, *J* = 9.0 Hz, H-2′ and 6′).

^{13}C NMR (CD_3OD): δ 62.5 0 4′-*O*-Glc-C-6), 62.52 (5-*O*-Glc-C-6), 71.22 (5-*O*-Glc-C-4), 71.34 (4′-*O*-Glc-C-4), 74.76 (5-*O*-Glc-C-2), 74.88 (4′-*O*-Glc-C-2), 77.33 (5-*O*-Glc-C-3), 77.98 (4′-*O*-Glc-C-3), 78.28 (4′-*O*-Glc-C-5), 78.59 (C-5″), 98.76 (C-8), 101.83 (4′-*O*-Glc-C-1), 104.40 (C-6), 104.90 (5-*O*-Glc-C-1), 107.49 (C-4a), 117.47 (2C, C-3′ and 5′), 126.44 (C-1′), 130.19 (2C, C-2′ and 6′), 139.20 (C-3),

144.86 (C-2), 160.14 (C-4′), 159.59 (C-8a), 159.81 (C-5), 165.14 (C-7), 173.95 (C-4), HR-FTICR-MS *m/z* 609.1470 $[M-H]^-$ (calculated for $C_{27}H_{29}O_{16}$ 609.1461).

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