Purification and Identification of Flavonoids from the Yellow Green Cocoon Shell (Sasamayu) of the Silkworm, *Bombyx mori*

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Three quercetin glycosides, quercetin 5-O-β-D-glucoside, quercetin 7-O-β-D-glucoside, and quercetin 4'-O-β-D-glucoside, and two kaempferol glycosides, kaempferol 5-O-β-D-glucoside and kaempferol 7-O-β-D-glucoside, along with their aglycones, quercetin and kaempferol, were isolated from an ethanolic extract of Sasamayu cocoon shells. The chemical structures were characterized by chemical and spectroscopic methods including UV spectrometry and HPLC-ESI-MS. The five flavonol glycosides of the shell are different structurally from those of the leaves of mulberry (*Morus alba*). It was suggested that potent antioxidative activity in the cocoon is mainly due to flavonoid compounds since free radical scavenging activity was found in the cocoon flavonoids identified here.

**Key words:** flavonoid; ethanolic cocoon shell extract; free radical scavenging activity; *Bombyx mori*

The silkworm *Bombyx mori* spins silk threads to make a cocoon shell, where some pigments coexist and accumulate in the layers of cocoon sericin. The coloring components are associated with phenolic compounds in the leaves of mulberry (*Morus alba*), the sole food for *B. mori* larvae. The content of cocoon coloring components varies depending on the *B. mori* strain. Some of the strains produce green cocoon shells containing at least nine fluorescent yellow compounds, five of which have been identified as flavonoid related compounds. Although the pattern of flavonoid accumulation in cocoons was found to be different genetically, little is known about the chemical structure of cocoon flavonoids.

The hybrid silkworm Sasamayu (142a × Daizo) produces silk threads containing yellow-green pigment. In our previous report, ethanolic extracts of cocoon shells (EECS) of the Sasamayu strain had potent antibacterial activity against both Gram-positive and Gram-negative bacteria. In addition, the EECS had strong antioxidative activity as well as tyrosinase inhibition. Judging from the UV absorption spectroscopic analysis of ethyl acetate extract from EECS, it was suggested that the antioxidative activity in cocoons is due to cocoon flavonoid compounds.

In this study, we purified seven flavonoids with antioxidative activity and their chemical structures were analyzed by HPLC-ESI-MS, UV spectroscopy, and enzymatic degradation.

Clean fresh cocoon shells of the silkworm *B. mori* (142a × Daizo) were chopped into small pieces, and samples of 100 g were extracted with 70% ethanol at 80°C for 24 h. The extract was filtrated, concentrated by evaporation, and lyophilized to yield EECS. The scheme for the extraction of cocoon flavonoid compounds is indicated in Fig. 1. EECS (2.4 g) dissolved in 100 ml of distilled water was washed twice with 50 ml of chloroform to remove lipids. The residual layer was extracted three times with ethyl acetate, and the ethyl acetate fractions were pooled together, concentrated by evaporation, and used for HPLC analysis. The ethyl acetate-insoluble fraction was further extracted three times with butanol to obtain the butanol soluble and insoluble fractions.

The ethyl acetate extract was purified using a C18 HPLC column (Wakosil-II-5C18 RS, 4.6 × 250 mm). The elution was done at room temperature by a linear gradient of 10 to 40% acetonitrile containing 0.1% TFA, at a flow rate of 1 ml/min. The chromatogram was monitored at 360 nm. Various eluted flavonoids were pooled separately and then measured for DPPH radical scavenging activity as described previously. HPLC-purified flavonoid peaks with the radical scavenging activity were further analyzed using an HPLC-ESI-MS (Finnigan TSQ 700). Ten-μl portions of each sample were separated on a Waters 626LC system using the Wakosil-II-5C18RS (4.6 × 250 mm) column used for the flavonoid purification and eluted by a linear gradient of 10 to 40% acetonitrile in 0.1% TFA, at a flow rate of 1.0 ml/min. ESI-MS was done in a positive ion mode with an ionization voltage of 4.5 kV. The source temperature was held at 260°C and acceleration voltage was 1200 V for all analyses.

Samples (0.5 mg) of flavonoid glycoside were hydrolyzed with 10 units of β-glucosidase (Sigma Corp.) in 0.5 ml of 20 mM acetate buffer (pH 5.0) at

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**Abbreviations:** EECS, ethanolic cocoon shell extract; TFA, trifluoroacetic acid; DPPH, α,α-diphenyl-β-picyrylhydrazyl

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30°C for 30 min to investigate sugar moieties and aglycons in glycosides. After incubation, the hydrolysate was lyophilized and dissolved in methanol to a final concentration of 1 mg/ml. The flavonol aglycons produced were separated by reversed-phase C18 HPLC as described for the flavonoids and identified by comparing the retention times with those from reference compounds. The sugar compounds were also identified by thin-layer chromatography.

In order to estimate the hydroxylation pattern of the flavonoids, two absorbance maxima at 300-385 nm (Band I) and 240-280 nm (Band II) in the presence or absence of reagents such as sodium methoxide (NaOMe), sodium acetate (NaOAc), and aluminum (III) chloride were measured by UV spectroscopy using a Jasco UV 2000 spectrometer.

EECS from Sasamayu cocoon shells showed considerable antioxidant activity, as reported previously by Yamazaki et al. The EECS was further fractionated according to the flow diagram depicted in Fig. 1. The yields of each fraction are shown in Table 1. In this fractionation method, most flavonoid compounds in the shell were extracted in the ethyl acetate fraction. The ethyl acetate fraction was the lowest degree of yield, with a value of 3.3%, and had a yellow color and a slightly spicy flavor. This fraction showed two absorbance maxima at 255 nm and 365 nm, characteristic absorption spectra for flavonol.

A typical chromatogram of flavonol in the ethyl acetate fraction at 360 nm is shown in Fig. 2. Based on DPPH radical scavenging activity experiments, flavonol compounds with the scavenging activity were separated in peaks 1, 2, 5, 6, 7, 10, and 11. Table 2 gives the data for UV absorbance maxima of the individual flavonol compounds in methanol solution. They had two characteristic flavonol UV absorbance maxima, one being in the range of 365-370 nm (Band I) and the other in the range of 253-265 nm (Band II).

Based on ESI-MS spectrometry, the seven peaks were considered to be pure, showing single [M+H]+ ions at m/z287 for peak 11, m/z303 for peak 10, and m/z449 for peaks 5 and 7, and m/z465 for peaks 1, 2, and 6.

Table 1. Yields of Extractive Fractions from Sasamayu Cocoon Shell

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Yield (% of EECS)</th>
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</thead>
<tbody>
<tr>
<td>Ethyl acetate solu.</td>
<td>3.3%</td>
</tr>
<tr>
<td>Butanol solu.</td>
<td>13.7%</td>
</tr>
<tr>
<td>Butanol insol.</td>
<td>73.3%</td>
</tr>
<tr>
<td><strong>Total recovery</strong></td>
<td><strong>90.3%</strong></td>
</tr>
</tbody>
</table>

Peaks 10 (m/z303) and 11 (m/z287) are probably quercetin and kaempferol, respectively. Based on their retention characteristics on C18 HPLC, peaks 10 and 11 were also confirmed to be such aglycons since they showed the same retention characteristics as respective standard compounds (data not shown).

Flavonol glycosides are more hydrophilic than the corresponding aglycons since the sugar moieties increase the hydrophilicity of the compounds. Since peaks 1, 2, 5, 6, and 7 were eluted earlier and more polar than quercetin and kaempferol, it is suggested that these peak fractions contain flavonol glycosides. In order to investigate the presence of a hexose sugar moiety, the five expected glycosides were hydrolyzed enzymatically with β-glucosidase to produce their corresponding aglycons and sugars. Based on C18 HPLC of the enzyme-released aglycons, peaks 1, 2, and 6 were confirmed as quercetin glycosides, while peaks 5 and 7 were identified as kaempferol glycosides (data not shown). Judging from thin-layer chro-
matography, they were found to be all glycosylated with D-glucose (data not shown).

The pattern of glycosylation was estimated by adding the shift reagents to each flavonol glycoside methanol solution. No glycosylation was observed at the C3 position in all of the flavonol glycosides.

Peak 1 and peak 7 are most likely quercetin 5-O-β-D-glucoside and kaempferol 5-O-β-D-glucoside, respectively. The sugar substitution of the flavonoid compounds is at the C5 position since the absorption spectra showed a hypsochromic shift compared with those of their corresponding aglycons. They had a bathochromic shift in the presence of NaOMe or NaOAc, indicating to have free hydroxyl groups at the C'4 and C7 positions.

Peak 2 and peak 5 are probably quercetin 7-O-β-D-glucoside and kaempferol 7-O-β-D-glucoside, respectively. Their free hydroxyl groups were confirmed to be at the C5 position since there was no bathochromic shift both in Band I and Band II peaks. The C7 position of each compound is sugar-substituted since the addition of NaOMe caused no changes in their absorption spectra.

Peak 6 appears to be quercetin 4'-O-β-D-glucoside. Based on UV spectroscopy, the presence of free hydroxyl groups was identified at the C3, C5, and C7 positions. The position of the sugar moiety was found to be C4' since the absorption spectrum of peak 6 showed a bathochromic shift in Band I and a decrease in the absorbance in the presence of NaOMe.

A list of molecular weights, absorption maxima, identifications and relative contents for each of the peaks is summarized in Table 2. Quercetin 7-O-β-D-glucoside is the most predominant flavonoid in the Sasamayu cocoon shell, with the relative content of 34.8%.

The B. mori silk fiber consists of fibroin and sericin, the two major proteins in silk, together with several minor components including both proteins and non-proteinous materials. Recently bioactive compounds such as protease inhibitor polypeptides have been isolated from B. mori cocoons and their presence was shown to protect silk protein from proteolytic degradation.5 In plants, flavonoids function as attractants of pollinators,5 UV-protectants,5 antimicrobial substances or phytoalexins,8 and signaling molecules9 in many species. Flavonoids in cocoon may thus be a protective agent and play an important role in protecting silk e.g. against microbial and UV degradation. Such compounds were also isolated, but relatively small in amount, from non-colored B. mori cocoon shells (unpublished data).

In this study, we elucidated the chemical structures of cocoon flavonol glycosides which are most likely insect metabolites closely related to plant phenolic compounds. Flavonol glycosides in plants occur usually as glycosides with sugars bound at the C3 position. The leaves of M. alba contain two major flavonol glycosides, isoquercitrin (quercetin 3-O-β-D-glucoside)10,11 and astragalin (kaempferol 3-O-β-D-glucoside).12 These glycosides were also found in M. alba ethanolic extract prepared in a similar way as in cocoon extract. However, the flavonol glycosides identified here were different structurally from the M. alba flavonol glycosides in terms of the position of glycosylation. Fujimoto and Hayashiya13,14 have demonstrated that isoquercitrin absorbed with the food is one of the most probable precursors of cocoon flavonoid glycosides, and that the alimentary canal cell is responsible for the biosynthesis of cocoon flavonoid glycosides. Since there are a variety of differently-colored cocoons, the accumulation of flavonoids in the silk gland, an organ where silk proteins are secreted, genetically depends on the alimentary canal cell capability to transport flavonoid into the blood.

Recently Kato et al.15 has reported that the sericin fraction is responsible for the antioxidant activity of the B. mori cocoon. We found the deproteinated sericin fraction to show the highest DPPH radical scavenging activity (unpublished data), and thus flavonoid compounds are also responsible for the antioxidant properties of B. mori cocoons.

References

2) Kurioka, A., Ishizaka, H., Yamazaki, M., and Endo,
Flavonoids in Sasamayu Cocoon Shell


