Anthocyanins from red flower tea (Benibana-cha), *Camellia sinensis*

Norihiko Terahara a,*, Yoshiyuki Takeda b, Atsushi Nesumi c, Toshibo Honda d

aDepartment of Food Science and Technology, College of Horticulture, Minami-Kyushu University, Takehara, Miyazaki 884-0003, Japan
bNational Research Institute of Vegetables, Ornamental Plants and Tea, Ministry of Agriculture, Forestry, and Fisheries, Makuzakazi 14041, Kagoshima 889-0032, Japan
cNational Research Institute of Vegetables, Ornamental Plants and Tea, Ministry of Agriculture, Forestry, and Fisheries, Kanaya-cho 2769, Shizuoka 428-8501, Japan
dInstitute of Medicinal Chemistry, Hoshi University, Shinagawa-ku, Tokyo 142-8501, Japan

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**Abstract**

Three anthocyanins were isolated from the leaves of red flower tea (Benibana-cha), *Camellia sinensis*, and their structures were determined by means of chemical and spectroscopic analyses. Two are the anthocyanins, delphinidin and cyanidin 3-0-β-D-galactosides, respectively. Whereas the third, delphinidin 3-0-β-D-(6-(E)-p-coumaryl)galactopyranoside was also contained in the flowers of Benibana-cha in different compositions. © 2001 Elsevier Science Ltd. All rights reserved.

**Keywords:** Benibana-cha; *Camellia sinensis*; Theaceae; Tea leaf; Delphinidin and cyanidin 3-0-β-D-galactosides; Acylated anthocyanin; Delphinidin 3-0-β-D-(6-(E)-p-coumaryl)galactopyranoside; Antioxidative activity

1. Introduction

Benibana-cha, originally grown in China, is a unique tea tree (*Camellia sinensis*) having red flowers and reddish green leaves. The red color is attributed to anthocyanin pigments contained in the plant. Thus the extract of Benibana-cha leaf is expected to contain highly bioactive substances, because it contains antioxidative anthocyanins (Tsuda et al., 1996; Noda et al., 1998; Obi et al., 1998) in addition to multi-biological functional catechins (Guo et al., 1996; Nanjo et al., 1996; Kumamoto and Senda, 1998; Kondo et al., 1999) which are normal green tea constituents. Since the anthocyanins had not yet been investigated, their structures were investigated using chemical and spectroscopic methods.

2. Results and discussion

From the leaves of Benibana-cha, we extracted the crude pigment which contained twelve or more Benibana-cha leaf anthocyanins (BCL-1 to 12). On purification of the crude extracts through several types of column chromatography, three anthocyanins 1, 2 and 3 were isolated as powders of trifluoroacetic acid (TFA) salts. On acid hydrolysis, 1 and 3 gave delphinidin (Dp), and 2 gave cyanidin (Cy) as aglycon, and all gave only galactose as the sugar by TLC analysis. On alkaline hydrolysis, 3 gave p-coumaric acid as an acyl component and 1 as a deacylated anthocyanin by HPLC analysis, but 1 and 2 were unchanged during similar treatment. In the UV-vis spectra of 1, 2 and 3, the values (20–27%) of $\frac{E_{440}}{E_{\text{vis}, \text{max}}}$ (a ratio of absorbances at 440 nm and visible maxima) suggested that 1, 2 and 3 were 3-O-substituted (Harborne, 1967). Observation of a bathochromic shift of their $\lambda_{\text{vis}, \text{max}}$ on addition of AlCl$_3$ solution suggested that the B-rings of 1, 2 and 3 were of the catechol- or pyrogallol-type. FABMS and ESI-TOFMS spectra of anthocyanins 1, 2 and 3 gave clear molecular-ion peaks at $m/z$ 465, 449 and 611 corresponding to the molecular formulae $C_{21}H_{21}O_{12}^+$, $C_{21}H_{21}O_{11}$ and $C_{30}H_{27}O_{14}^+$, respectively. Therefore the compositions of 1, 2 and 3 were deduced as Dp mono-hexoside, Cy mono-hexoside, and Dp-p-coumaryl-mono-hexoside, respectively. Anthocyanin 2 was chromatographically identical to cyanidin 3-0-β-D-galactoside by comparison with the authentic anthocyanin from berries of *Fatsia japonica* (Terahara et al., 1992). (Fig. 1)

Determination of detailed chemical structures of 1 and 3 were performed through $^1$H-NMR analyses using
DQF-COSY and DIFNOE methods as previously reported (Terahara et al., 1998). The assignments are depicted in Table 1. In the low magnetic field (δH 6–9 ppm), four singlets of 1 and 3 were assignable to protons of Dp having a symmetric B-ring. In 3, two pairs of characteristic doublets with large coupling constants (J=16 Hz) were allocated to the olefinic α- and β-protons of the (E)-hydroxycinnamoyl moiety. In the high magnetic field (δH 3–6 ppm), the assignment of galactosyl protons was facilitated by DQF-COSY and DIFNOE spectra. As the anomic protons in sugars of 1 and 3 were characteristic doublets shifted to lower field with large coupling constants (7–8 Hz) and the ring protons other than the 4-methine proton had large coupling constants (7–9 Hz), all sugars have the β-D-galactopyranosyl configuration (Terahara et al., 1992). The 5-methylene and 6-methylene protons in 3 were observed at lower magnetic field (the chemical shift differences, ΔδH from +0.4 to +0.5 ppm) as compared with those of 1, indicating the 6-OH position of 3 was acylated. Attachment positions of sugars in 1 and 3 were clearly determined by DIFNOE spectra. Cross-peaks of intense NOEs between Dp-4-H and Gal-1-H, proved that Dp-3-OH was glycosylated with Gal. In conclusion, Benibana-cha leaf major anthocyanins 1 and 3 were identified as delphinidin 3-O-β-D-galactopyranoside and delphinidin 3-O-β-D-(6-O-(E)-p-coumaryl)galactopyranoside, a new anthocyanin, respectively. Pigment 2 was identified as cyanidin 3-O-β-D-galactopyranoside.

The crude extract from the leaves of Benibana-cha contains more than twelve anthocyanins, in which anthocyanin 1 is of highest concentration, 2 and 3 followed by those of anthocyanins. These pigments are also contained in the flowers in different composition, in which the order of contents is 2 > 1 > 3.

So far as we know, tricetinidin has been only found in a manufactured tea (Roberts and Williams, 1958). It is formed as a result of oxidation of (−)-epigallocatechin gallate. Therefore, it is the first time that intact anthocyanins were found in tea leaves.

The leaf extract of Benibana-cha is expected to be used as high functional natural colorants for food or other purposes, because it contains antioxidative anthocyanins (Tsuda et al., 1996; Noda et al., 1998; Obi et al., 1998) in addition to biologically multi-functional catechins (Guo et al., 1996; Nanjo et al., 1996; Kumamoto and Sonda., 1998; Kondo et al., 1999).

3. Experimental

3.1. General experimental procedures

All reagents used were special or HPLC grades purchased from Kishida Chemical Co. and Wako Chemical Ind., Japan. TLC was carried out as previously reported (Terahara et al., 1992). HPLC was done on an L-6200 Intelligent pump system (Hitachi Co., Japan). The analytical HPLC was run on a Luna column (4.5 i.d.×150 mm, Phenomenex Co., USA) at 35°C with monitoring at 520 nm. The flow rate was 1.0 ml min⁻¹ with a linear gradient elution for 80 min from 0% to 40% solvent B (1.5% H₃PO₄, 20% HOAc, 25% MeCN in H₂O, v/v) in solvent A (1.5% H₃PO₄ in H₂O, v/v). Preparative HPLC was performed on an Inertsil ODS 5 (Benibana-cha) leaves (Table 1) leaves [δH ppm from internal standard (CH₃)₄Si in DMSO-d₆;CF₃COOD (9:1)]

<table>
<thead>
<tr>
<th>Assignment</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delphinidin</td>
<td>4</td>
<td>8.87 †</td>
<td>8.74 †</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>6.92 †</td>
<td>6.82 brs</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>6.71 s</td>
<td>6.63 brs</td>
</tr>
<tr>
<td></td>
<td>2′, 6′</td>
<td>7.76 s</td>
<td>7.67 s</td>
</tr>
<tr>
<td>Galactosyl</td>
<td>1</td>
<td>5.30 d (7.9)</td>
<td>5.32 d (7.6)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3.92 brt (8.7)</td>
<td>3.83 t (8.5)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3.60 dd (3.3, 8.8)</td>
<td>3.61 dd (3.1, 7.9)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>3.66 dd (3.3, 5.5)</td>
<td>3.83 brd (3.1)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>3.62 m</td>
<td>4.11 m</td>
</tr>
<tr>
<td></td>
<td>6a</td>
<td>3.79 dd (3.4, 8.2)</td>
<td>4.16 dd (2.9, 8.5)</td>
</tr>
<tr>
<td></td>
<td>6b</td>
<td>3.86 brd (3.4)</td>
<td>4.36 dd (2.4, 9.2)</td>
</tr>
<tr>
<td>p-Coumaryl</td>
<td>2, 6</td>
<td>7.35 d (8.2)</td>
<td>7.35 d (8.2)</td>
</tr>
<tr>
<td></td>
<td>3, 5</td>
<td>6.76 d (8.5)</td>
<td>6.76 d (8.5)</td>
</tr>
<tr>
<td></td>
<td>α</td>
<td>6.26 d (15.9)</td>
<td>6.26 d (15.9)</td>
</tr>
<tr>
<td></td>
<td>β</td>
<td>7.40 d (15.9)</td>
<td>7.40 d (15.9)</td>
</tr>
</tbody>
</table>

* Values in parentheses indicate coupling constants (J in Hz). Chemical shift differences: ΔδH=(δH of 3)−(δH of 1).

Fig. 1. The structures of anthocyanins 1, 2 and 3 from leaf of red flower tea (Benibana-cha), Camellia sinensis.
column (20 i.d.×250 mm, GL Sciences Inc., Japan) at 7 ml min⁻¹ by an isocratic elution using mixtures of solvent A (HOAc:H₂O=15:85, v/v) and solvent B (HOAc:MeCN:H₂O=15:30:55, v/v). A:B=75:25 at 520 nm. UV-vis spectra were recorded on MPS-2000 spectrophotometer (Shimadzu Co., Ltd, Japan) in 0.01% HCl–MeOH. FABMS spectra were measured on JMS SX-102 (JEOL Co., Ltd) in MeOH with Magic Bullet or m-nitrobenzyl alcohol as a matrix with a positive mode. ESI-TOFMS spectra were measured on Mariner Biospectrometry Workstation (PerSeptive Biosystems Inc., USA) in 1% HOAc–50% MeCN with no matrix in the positive mode. 1H (500 MHz) NMR were run on a JMN spectrometry Workstation (PerSeptive Biosystems Inc., USA) in 1%HOAc–50%MeCN with no matrix in the solvent A (HOAc:H₂O=15:85, v/v) and solvent B (HOAc:MeCN:H₂O=15:30:55, v/v), A:B=75:25.

### 3.3. Isolation and preparation of pigments

The dried leaves were used for our experiment. The dried leaves were harvested in April 1997, and dried by warm wind overnight. The dried leaves were used for our experiment.

### 3.4. Chemical analyses

Acid and alkaline hydrolyses of isolated pigments were performed according to previous methods (Terahara et al., 1992).

### 3.5. Pigment I (Delphinidin 3-O-β-D-galactopyranoside)

FABMS: m/z 465 [M=C₂₁H₂₂O₁₂] with Magic Bullet or m-nitrobenzyl alcohol as a matrix, 487 [M+Na⁺] with Na⁺ + Magic Bullet; ESI-TOFMS: m/z 465 [M=C₂₁H₂₂O₁₂]; UV-vis λmax (0.01% HCl–MeOH) nm: 540 (bathochromic shift 15 nm with AlCl₃), 277. E₄⁴⁰/E₅₄⁰= 277/540 = 20%; 1H NMR data: see Table 1.

### 3.6. Pigment 2 (Cyanidin 3-O-β-D-galactoside)

FABMS: m/z 449 [M=C₂₁H₂₁O₁₁] with Magic Bullet; ESI-TOFMS: m/z 449 [M=C₂₁H₂₁O₁₁]; UV-vis λmax (0.01% HCl–MeOH) nm: 529 (bathochromic shift 20 nm with AlCl₃), 273, E₄⁴⁰/E₅₂⁹= E₄⁴⁰/E₅₂⁹ = 25%.

### 3.7. Pigment 3 (Delphinidin 3-O-β-D-(6′-E)-p-coumaryl)galactopyranoside)

FABMS: m/z 611 [M=C₃₀H₂₇O₁₄] with Magic Bullet; ESI-TOFMS: m/z 611 [M=C₃₀H₂₇O₁₄]; UV-vis λmax (0.01% HCl–MeOH) nm: 541 (bathochromic shift 14 nm with AlCl₃), 310, 271, E₄⁴⁰/E₅₄₁= E₄⁴⁰/E₅₄₁ = 176%; 1H NMR data: see Table 1.

### References


