Rapid Paper

Structure-related Emission Spectrometric Analysis of the Chemiluminescence of Catechins, Theaflavins and Anthocyanins

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This study for the first time achieved an emission spectrometric analysis of the chemiluminescence of flavonoids in the presence of hydrogen peroxide, acetaldehyde and horseradish peroxidase, and revealed that the maximum emission wavelengths (Emax) strictly differ among catechins (Emax 630 nm), theaflavins (Emax 690 nm) and anthocyanins (Emax 675 nm) according to their chemical structures. This technique enabled the direct incorporation of dietary tea catechin into rat intestinal mucosal cells to be spectrometrically confirmed.

Key words: catechins; theaflavins; anthocyanins; chemiluminescence; emission spectrometry

The pathophysiological functions of food flavonoids in humans have recently been receiving much attention.¹² In order to investigate the metabolic fate of flavonoids, we have already established a new methodology, chemiluminescence detection high-performance liquid chromatography (CL-HPLC).³ This new method enables the determination of flavonoids in biological samples, i.e., human plasma and rat liver, at a level as low as a few picomoles with high selectivity. By using this method, the absorption and metabolism of tea catechins in humans and in rats have been extensively studied.³⁻⁵ This system induces chemiluminescence by the reaction of flavonoids with hydrogen peroxide, acetaldehyde and horseradish peroxidase and employs post-column detection.³ However, the emission spectrometric characterization of the chemiluminescence of flavonoids has never been accomplished.

In the present study, we carried out a further chemiluminescence emission spectrometric analysis of such food flavonoids as catechins, theaflavins and anthocyanins, and found that these flavonoids showed characteristic maximum emission wavelengths solely depending on their chemical structures. The direct incorporation of tea catechin into the intestinal mucosal cells of a rat that had ingested tea catechin was confirmed by employing this emission spectrometric assay.

Materials and Methods

Reagents. (−)-Epigallocatechin-3-gallate (EGCg), (−)-epigallocatechin (EGC) and (−)-epicatechin (EC) were presented by Taiyo Kagaku Co. (Yokkaichi, Japan), and theaflavin digallate (TF-2) and theaflavin (TF) likewise by Mitsui Norin Co. (Fujieda, Japan). Delphinidin (DEL), cyanidin (CYA), cyanidin 3-glucoside (CYA-G), gallic acid and caffeic acid were purchased from Funakoshi Co. (Tokyo, Japan), and curcumin and ascorbic acid were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). α-Tocopherol was from Eisai Co. (Tokyo, Japan). All other chemicals used were at least of reagent grade.

Detection and spectrometric analysis of chemiluminescence. The chemiluminescence measurements and emission spectrometric analysis were carried out with a CLA-SP2 chemiluminescence spectrum analyzer (Tohoku Electronic Ind. Co., Sendai, Japan) in an air atmosphere at room temperature for 500 s. A 1.2 ml volume of 8.2 M acetaldehyde in a 50 mM phosphate buffer (pH 7.4, containing 108 mg of horseradish peroxidase/L) and a 400 µl of 8.8 M hydrogen peroxide aqueous solution were mixed in a 4.0-ml quartz cell (1 × 1 × 4 cm), 1.5 µmol of a sample flavonoid or other phenolic dissolved in 400 µl of methanol/water (2:8 v/v; containing 0.1% phosphoric acid) was added and the chemiluminescence was analyzed. The analysis of curcumin and α-tocopherol used only methanol for dilution. The background count, in the absence of a flavonoid, was 3 × 10⁶ counts/500 s. Resolution of the spectrometry was 4 nm in the wavelength region between 270 and 870 nm.

Animal trial. Male Sprague-Dawley rats (300 g in body wt) were orally dosed with EGCg (23 mg/100 g of body wt, dissolved in 2 ml of distilled water, a single administration). Sixty minutes after the administration, the small intestinal mucosa was separated and well washed repeatedly with 0.15 M saline to remove EGCg adhering to the surface. A 400 µl volume of a 50% (w/v) mucosal cell homogenate in 0.15 M saline was prepared. The chemiluminescence emission spectrum, in the presence of hydrogen peroxide, acetaldehyde and horseradish peroxidase, was recorded to confirm the direct incorporation and occurrence of EGCg in the mucosal cells.

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Abbreviations: Emax, maximum emission wavelength; CL-HPLC, chemiluminescence high-performance liquid chromatography; EGCg, (−)-epigallocatechin-3-gallate; EGC, (−)-epigallocatechin; EC, (−)-epicatechin; TF-2, theaflavin digallate; TF, theaflavin; DEL, delphinidin; CYA, cyanidin; CYA-G, cyanidin 3-glucoside.
Results and Discussion

Chemiluminescence of the flavonoids

Figure 1 shows a typical chemiluminescence phenomenon for EGCg (990 × 10^4 counts/500 s) in the presence of hydrogen peroxide, acetaldehyde and horseradish peroxidase. Other flavonoids showed almost the same chemiluminescence reaction, although the intensity significantly varied depending on their chemical structures (Table I). The chemiluminescence intensity of the flavonoids examined was in the order of EGCg > EGC > TF-2 > DEL > CYA-G > CYA > TF > EC.

The chemiluminescence reaction of such theaflavins as TF-2 and TF was thus confirmed in the present study. The results indicate that the chemical structure most responsible for emitting strong chemiluminescence is three hydroxyl groups in the B-aromatic ring of the flavonoid. This is similar to the finding in the absence of horseradish peroxidase by Yoshiki et al. Since EC, CYA and CYA-G have only two hydroxyl groups in the B-aromatic ring, their chemiluminescence was rather low. The strong chemiluminescence observed in TF-2 could be ascribed to the presence of two gallic acid molecules. Gallic acid itself also revealed chemiluminescence, while hydroxyl compounds like tocopherol and ascorbic acid did not show any chemiluminescence.

Maximum emission wavelength of the flavonoids

EGCg chemiluminescence has a wide emission wavelength region of 510–870 nm with an emission maximum (Emax) of 630 nm (Fig. 2a). All the catechins (EGCg, EGC and EC) examined showed the same Emax at 630 nm. Theaflavins such as TF-2 (Fig. 2b) and TF gave Emax at 690 nm, and anthocyanins such as DEL (Fig. 3), CYA and CYA-G revealed Emax at 675 nm.

These data clearly demonstrate the strict difference in Emax among such flavonoids as catechins, theaflavins and anthocyanins. Such differences in Emax would depend on the different oxidizability of the central pyran ring in the basic structure of the flavonoid molecule, and not on the number of hydroxyl groups in the aromatic rings. In connection to this, we have previously explained the chemiluminescence reaction as follows:\textsuperscript{30}

\[
P(\text{polyphenol}) + \text{HOOH} \rightarrow \text{PH-OOH}
\]

(oxygenated intermediate of polyphenol)

![Fig. 2. Emission Spectra of the Chemiluminescence of (-)-Epigallocatechin-3-gallate (EGCg) (a) and Theaflavin Digallate (TF-2) (b) in the Presence of Hydrogen Peroxide, Acetaldehyde and Horseradish Peroxidase.](image)

The analytical conditions are described in the text.

Fig. 1. Chemiluminescence of (-)-Epigallocatechin-3-gallate (EGCg) in the Presence of Hydrogen Peroxide, Acetaldehyde and Horseradish Peroxidase.

The color expresses the intensity as white (most intense) > red > yellow > green > blue (very weak) > black. a, control (no EGCg); b, EGCg added. See the text for details.
Table I. Maximum Emission Wavelength (Emax) of Chemiluminescence from Flavonoids in the Presence of Hydrogen Peroxide, Acetaldehyde and Horseradish Peroxidase

<table>
<thead>
<tr>
<th>Compound</th>
<th>Substituent position</th>
<th>Emax (nm)</th>
<th>Chemiluminescence intensity (x 10⁶ counts/500 sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catechins</td>
<td>(R1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epigallocatechin-3-gallate (EGCg)</td>
<td>(R2)</td>
<td>OH Ga⁸</td>
<td>630</td>
</tr>
<tr>
<td>Epigallocatechin (EGC)</td>
<td></td>
<td>OH H</td>
<td>630</td>
</tr>
<tr>
<td>Epicatechin (EC)</td>
<td></td>
<td>H H</td>
<td>630</td>
</tr>
<tr>
<td>Theaflavins</td>
<td>(R3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Theaflavin digallate (TF-2)</td>
<td>(R4)</td>
<td>Ga Ga</td>
<td>690</td>
</tr>
<tr>
<td>Theaflavin (TF)</td>
<td></td>
<td>H H</td>
<td>690</td>
</tr>
<tr>
<td>Anthocyanins</td>
<td>(R5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Delphinidin (DEL)</td>
<td>(R6)</td>
<td>OH H</td>
<td>675</td>
</tr>
<tr>
<td>Cyanidin (CYA)</td>
<td></td>
<td>H H</td>
<td>675</td>
</tr>
<tr>
<td>Cyanidin 3-glucoside (CYA-G)</td>
<td></td>
<td>H Glc⁹</td>
<td>675</td>
</tr>
<tr>
<td>Other phenolics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gallic acid</td>
<td></td>
<td></td>
<td>670</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td></td>
<td></td>
<td>670</td>
</tr>
<tr>
<td>Curcumin</td>
<td></td>
<td></td>
<td>640</td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>(Ascorbic acid)</td>
<td>No chemiluminescence</td>
<td>No chemiluminescence</td>
</tr>
</tbody>
</table>

⁸ galloyl.
⁹ glucose.
The chemical structures are given below, and R1–R6 are substituted as given above.

![Chemiluminescence Emission Spectra of Flavonoids](image)

Direct emission spectrum of EGCg in small intestinal mucosal cells of a rat after ingesting tea catechin

Figure 4 shows that the emission spectrum (Emax 630 nm) of small intestinal mucosal cells prepared from the rat 60 min after a single oral administration of EGCg (23 mg/100 g of body wt of the rat) was identical to the emission spectrum of authentic EGCg. This finding demonstrates that EGCg orally ingested by the rat was directly incorporated into the intestinal mucosal cells. This agrees well with our previous findings⁷ on the accumulation of dietary catechins in intestinal mucosa as measured by CL-HPLC, and is important evidence for the potent anticarcinogenic and antioxidative actions of EGCg in preventing the development of colon cancer.⁷

In conclusion, this study has explained for the first time the difference in the maximum emission
Fig. 3. Emission Spectrum of the Chemiluminescence of Delpinidin (DEL) in the Presence of Hydrogen Peroxide, Acetaldehyde and Horseradish Peroxidase.
The analytical conditions are described in the text.

Fig. 4. Emission Spectrum of the Chemiluminescence of Small Intestinal Mucosa from a Rat that Had Orally Ingested (−)-Epigallocatechin-3-gallate (EGCg).
a, control rat (untreated); b, EGCg-treated rat. The analytical conditions are described in the text.

wavelengths of chemiluminescence from such flavonoids as catechins, theaflavins and anthocyanins, and further demonstrated the direct incorporation of food catechin into rat intestinal mucosal cells by applying an emission spectrometric analysis. This spectrometric analysis will be a powerful tool for investigating the composition and function of flavonoids present in foods and biological samples.

Acknowledgments
The samples of EGCg, EGC and EC presented by Taiyo Kagaku Co. (Yokkaichi, Japan), and of TF-2 and TF by Mitsui Norin Co. (Fujieda, Japan) are gratefully acknowledged.

References