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Investigation of epigallocatechin-3-*O*-caffeoate and epigallocatechin-3-*O*-*p*-coumaroate in tea leaves by LC/MS-MS analysis

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ABSTRACT

(−)-Epigallocatechin-3-*O*-gallate (EGCG), the major catechin present in green tea, exhibits potent antioxidant activity. We thereby investigated the presence of unknown components bearing the (−)-epigallocatechin (EGC) moiety in fresh tea leaf samples. Initially, liquid chromatography tandem mass spectrometry (LC-MS/MS) was employed to examine fresh tea leaves of the Yabukita, the most popular tea cultivar in Japan, which suggested the presence of the EGC phenylpropanoid derivatives, (−)-epigallocatechin-3-*O*-*p*-coumaroate (EGC*p*CA) and (−)-epigallocatechin-3-*O*-caffeoate (EGCCA). The structures of the detected EGC*p*CA and EGCCA were then confirmed by LC-MS/MS using synthesized EGC*p*CA and EGCCA as standards. In addition, EGC*p*CA and EGCCA were evaluated for their antioxidant activity in the ORAC (oxygen radical antioxidant capacity) and DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assays, where EGCCA (8.60 μmol TE/μmol, TE = Trolox equivalents) exhibited a stronger antioxidant activity than EGCG (5.52 μmol TE/μmol) in the ORAC assay. Finally, EGC*p*CA and EGCCA were quantitated in several tea leaf samples using LC-MS/MS, and it was found that these compounds were present in lower quantities (EGC*p*CA, 16.8–345.8 μg/g, EGCCA, 4.3–75.1 μg/g in the dry tea leaves) than the major catechins. In this study, we found the potent antioxidant EGCCA using LC-MS/MS and revealed its wide existence in various tea leaves.

1. Introduction

Tea (Camellia sinensis L.) is consumed in many countries, with green tea consumption being particularly common in Asian countries. (−)-Epigallocatechin-3-*O*-gallate (EGCG) is the major catechin present in green tea, and is formed by the condensation of (−)-epigallocatechin (EGC) with gallic acid at the 3 position. EGCG is of particular interest because it exhibits a range of biological activities, including anti-hypertensive, anti-allergenic, and anti-cancer properties, with more recent studies suggesting that it has the potential to prevent Alzheimer's disease (Bagchi, 2016; Wei et al., 2016). In addition to the gallic acid derivatives, *O*-methyl gallate and mono-phenylpropanoid derivatives have also been detected in nature, and their biological activities have been reported. For example, (−)-epigallocatechin-3-*O*-(3-*O*-methyl) gallate (EGCG3″Me) reduces seasonal allergic rhinitis symptoms both in vivo and in vitro (Maeda-Yamamoto, Ema, & Shibuichi, 2007; Suzuki, Yoshino, Maeda-Yamamoto, Miyase, & Sano, 2000), it exhibits a stronger activity than EGCG in the inhibition of the angiotensin-converting enzyme in vitro (Kurita, Maeda-Yamamoto, Tachibana, & Kamei, 2010), and it has a higher bioavailability than EGCG in rats (Oritani et al., 2013). Furthermore, (−)-epigallocatechin-3-*O*-*p*-coumaroate (EGC*p*CA), which has been elucidated through chemical reactions in addition to MS and NMR analyses, exhibits a stronger yeast alcohol dehydrogenase inhibitory activity (Manir, Kim, Lee, & Moon, 2012) than EGCG. It was therefore assumed that modification or replacement of the gallate moiety in EGCG influences the bioactivity of the catechins. While the biological activities of a number of compounds which have EGC moiety, have been reported, other compounds such as (−)-epigallocatechin-3-*O*-caffeoate (EGCCA) remain unexamined, despites their structures having been elucidated (Hashimoto, Nonaka, & Nishioka, 1989).

Thus, presence of EGC derivatives in tea leaves was investigated in this study. Initially, liquid chromatography tandem mass spectrometry (LC-MS/MS) will be carried out to detect any EGC derivatives, such as

Abbreviations: DMAP, *N*,*N*-dimethylaminopyridine; DPPH, 2,2-diphenyl-1-picrylhydrazyl; EDCI-HCl, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride; EGC, (−)-epigallocatechin; EGCCA, (−)-epigallocatechin-3-*O*-caffeoate; EGCG, (−)-epigallocatechin-3-*O*-gallate; EGCG3″Me, (−)-epigallocatechin-3-*O*-(3-*O*-methyl) gallate; EGC*p*CA, (−)-epigallocatechin-3-*O*-*p*-coumaroate; MRM, multiple reaction monitoring; NsCl, 2-nitrobenzenesulfonyl chloride; ORAC, oxygen radical antioxidant capacity; TE, trolox equivalents

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the EGC phenylpropanoid derivatives, present in fresh tea leaves of the Yabukita, the most popular tea cultivar in Japan. We will also carry out structure elucidation and examine the antioxidant activities of any EGC derivatives detected in the tea leaves.

2. Materials and methods

2.1. Chemicals

Caffeic acid (> 98.0%), *p*-coumaric acid (> 98.0%), 2-nitrobenzenesulfonyl chloride (> 95.0%), and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (> 98.0%) were obtained from Tokyo Chemical Industry (Tokyo, Japan), while epigallocatechin (> 95.0%) was purchased from Sigma-Aldrich Japan (Tokyo, Japan). High performance liquid chromatography (HPLC) grade acetonitrile (> 99.8%), special grade acetic acid (> 99.7%), special grade triethylamine (> 99.0%), special grade 4-dimethylaminopyridine (> 99.0%), special grade sodium sulfate (> 99.0%), special grade allyl alcohol (> 99.0%), special grade dimethyl sulfoxide (> 99.0%), 1st grade cesium carbonate (95.0–102.0%), and Wakogel® FC-40 (20–40 μm, > 70%) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). LC-MS grade acetonitrile (> 99.9%), LC-MS grade distilled water, HPLC grade formic acid (> 98.0%), special grade ethyl acetate (> 99.5%), and special grade dichloromethane (> 99.5%) were obtained from Kanto Chemical Co., Inc. (Tokyo, Japan).

2.2. Plant materials

Fresh green tea leaves of the Yabukita cultivar, Okumidori cultivar, and Assam cultivar were obtained from nursery trees purchased from a garden center in Japan and were freeze-dried. All other tea leaves were obtained from the Chiyonoen Tea Farm (i.e., Okumidori cultivar, Yabukita cultivar, Saemidori cultivar, and Okuyutaka cultivar: non-fermented tea) and from HAMASA-EN Co., Ltd. (i.e., Assam black tea, Uva, Darjeeling tea: fermented tea, Blue tea: semi-fermented tea, and Pu'ercha: after-fermented tea). All samples were powdered with mortar and pestle.

2.3. Sample preparation

Each dried or powdered tea leaf sample (~600 mg) was added to an 80% (v/v) ethanol/water solution (20 mL) and extracted twice for 1 h at 100 °C (reflux). The individual aqueous ethanol extracts were then evaporated to remove ethanol and freeze-dried to remove water. The resulting powdered extracts were added to a 90% (v/v) acetonitrile/water solution (600 μL) and extracted three times. The aqueous acetonitrile extracts were then transferred into 2 mL measuring flasks and the volumes made up to 2 mL using the 90% (v/v) acetonitrile/water solution. Finally, these solutions were diluted with a 0.1% (v/v) formic acid/water solution, and filtered through a 0.22 μm Millipore membrane filter prior to chromatographic analysis.

2.4. Mass spectrometric determination of the EGC derivatives present in the tea extracts

The extracted samples were analyzed using a Shimadzu Prominence Ultrafast Liquid Chromatograph (UFLC) system equipped with a Kinetex 2.6 μm C18 100 Å ODS column (100 mm × 2.1 mm i.d.; Phenomenex, Torrance, CA, USA). Elution was carried out at 40 °C, using a 0.1% (v/v) formic acid/water solution as mobile phase A, and a 0.1% (v/v) formic acid/acetonitrile solution as mobile phase B. A flow rate of 0.2 mL/min was employed and the gradient conditions were as follows: 0–5 min, 5% B; 5–37 min, 5–45% B; and 37–37.5 min 45–95% B, followed by an isocratic plateau for 5 min and return to the initial conditions (i.e., 5% B). The injection volume was 2.0 μL. Mass spectrometric analysis was performed using a 3200 QTRAP system (AB SCIEX, Framingham, MA, USA) with an electrospray ionization source. Electrospray ionization tandem mass spectrometry was performed in negative polarity mode using the following settings: Curtain gas, 40 psi; nebulizer gas, 50 psi; turbo gas, 80 psi; capillary temperature, 600 °C; ion spray voltage, − 4.5 kV; declustering potential, − 30 V; collision energy, − 50 to − 20 V (Setoguchi et al., 2014). The instrument was used in the tandem mass mode with a negative precursor ion scan. Following data acquisition by precursor ion scanning, the instrument was switched to product ion scan mode.

2.5. NMR measurements

All ^{1}H and ^{13}C NMR spectra were recorded in CD_3OD using a Bruker Avance 500 MHz NMR spectrometer (at 500 and 125 MHz, respectively) (Bruker, Yokohama, Japan). Chemical shifts are given in δ (ppm) relative to tetramethylsilane as an internal standard (bs = broad singlet, s = singlet, d = doublet, and dd = double doublet).

2.5.1. EGCpCA

^{1}H NMR (500 MHz, CD_3OD): 2.82 (1H, dd, J = 17.4, 2.6 Hz), 2.95 (1H, dd, J = 17.4, 4.7 Hz), 4.93 (1H, bs), 5.44 (1H, m), 5.94 (1H, d, J = 2.2 Hz), 5.95 (1H, d, J = 2.2 Hz), 6.23 (1H, d, J = 15.9 Hz), 6.50 (2H, s), 6.75 (2H, d, J = 8.7 Hz), 7.40 (2H, d, J = 8.7 Hz), 7.48 (1H, d, J = 15.9 Hz); ^{13}C NMR (125 MHz, CD_3OD): 26.7, 69.8, 78.5, 95.8, 96.5, 99.3, 106.8 (2C), 115.2, 116.7 (2C), 127.2, 130.7, 131.3 (2C), 133.8, 146.7 (2C), 146.8, 157.1, 157.8, 157.9, 161.1, 168.6. MS (ESI): m/z 451.1 $(M - H)^-$.

2.5.2. EGC-3-O-caffeoate (EGCCA)

^{1}H NMR (500 MHz, CD_3OD): 2.81 (1H, dd, J = 17.4, 2.2 Hz), 2.95 (1H, dd, J = 17.4, 4.8 Hz), 4.93 (1H, bs), 5.44 (1H, m), 5.94 (1H, d, J = 2.4 Hz), 5.96 (1H, d, J = 2.4 Hz), 6.17 (1H, d, J = 15.8 Hz), 6.49 (2H, s), 6.72 (1H, d, J = 8.2 Hz), 6.89 (1H, dd, J = 8.2, 1.9 Hz), 6.98 (1H, d, J = 1.9 Hz), 7.41 (1H, d, J = 15.8 Hz); ^{13}C NMR (125 MHz, CD_3OD): 26.7, 69.8, 78.4, 95.8, 96.5, 99.4, 106.8 (2C), 115.1, 115.2, 116.4, 123.1, 127.8, 130.7, 133.8, 146.6, 146.7 (2C), 147.2, 149.4, 157.1, 157.8, 157.9, 168.6. MS (ESI): m/z 467.6 $(M - H)^-$. HR-ESIMS: $(M - H)^-$ m/z 467.0983 (m/z 467.0978 calcd for $C_{24}H_{19}O_{10}$).

2.6. 1,1-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity assays

The radical scavenging activities of the isolated phenylpropanoids were evaluated against the DPPH radical using the conventional assay method (i.e., a modification of the method reported by Sano, Sugiyama, Ito, Katano, & Ishihata, 2011). Two different solutions were prepared, where solution A contained 40–2000 μM serial dilutions of the samples of interest in a mixture of dimethyl sulfoxide (DMSO) and ethanol (1:3), and solution B was 0.1 mM DPPH in ethanol. Solution A (20 μL) was mixed thoroughly with solution B (180 μL) in a test tube and stored at 20–25 °C for 20 min. The absorbance of the resulting solution was then measured at 520 nm using an Emax Precision Microplate Reader (Molecular Devices, USA). All samples were analyzed in triplicate and the mean values were calculated. These obtained values for the DPPH radical scavenging activities of the samples were compared with that of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), which was employed as a control.

2.7. Oxygen radical absorbance capacity (ORAC) assays

The ORAC values of the EGC derivatives were evaluated against the 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) radical in accordance with the conventional method (Watanabe et al., 2012). EGCG, EGC*p*CA, EGCCA, and Trolox were dissolved in DMSO (serial dilutions of 0.16–1.25 mM for EGCG, EGC*p*CA, and EGCCA; 0.31–5.0 mM