DOI:10.1021/jf100493s

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Cloning of a Novel *O*-Methyltransferase from *Camellia sinensis* and Synthesis of *O*-Methylated EGCG and Evaluation of their Bioactivity

Masanobu Kirita,*,† Daiki Honma,† Yoshihisa Tanaka,† Shinya Usui,‡ Toshihiko Shoji,† Manabu Sami,† Toyokazu Yokota,† Motoyuki Tagashira,† Atsuya Muranaka,‡ Masanobu Uchiyama,‡ Tomomasa Kanda,† and Mari Maeda-Yamamoto§

†Research Laboratories for Fundamental Technology of Food, Asahi Breweries Limited, 1-21, Midori-1, Moriya-shi, Ibaraki 302-0106, Japan, ‡Advanced Elements Chemistry Laboratory, The Institute of Physical and Chemical Research, RIKEN, 2-1 Hirosawa, Wako-shi, Saitama 351-0198, Japan, and §National Institute of Vegetable and Tea Science, National Agriculture and Food Research Organization, 2769 Kanaya, Shimada-shi, Shizuoka 428-8501, Japan

The gene of a novel *O*-methyltransferase was isolated from tea cultivars (*Camellia sinensis* L.). Using the recombinant enzyme, *O*-methylated (−)-epigallocatechin-3-*O*-gallate (EGCG) in all cases were synthesized. EGCG and the synthesized *O*-methylated EGCGs including (−)-epigallocatechin-3-*O*-(3-*O*-methyl)-gallate (EGCG3″Me), (−)-epigallocatechin-3-*O*- (4-*O*-methyl)-gallate(EGCG4″Me), (−)-epigallocatechin-3-*O*-(3,5-*O*-dimethyl)-gallate (EGCG3″,5″diMe), and (−)-3-*O*-methyl-epigallocatechin-3-*O*-(3,5-*O*-dimethyl)-gallate (EGCG3′,3″,5″triMe) were assayed using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay and antibacterial activity. EGCG was the most effective of the *O*-methylated EGCGs. The antiallergic effects of EGCG and the other *O*-methylated EGCGs were measured by conducting histamine release assays using bone marrow-derived mouse mast cells, and the order of potency was EGCG3′,3″,5″triMe = EGCG3″,5″diMe > EGCG3″Me > EGCG. These results indicated that reducing the number of hydroxyl groups decreases the effectiveness of DPPH radical scavenging and antibacterial activity. In contrast, the inhibition of histamine release was potentiated by an increase in the number of methyl groups in EGCG, especially in the galloyl moiety.

KEYWORDS: *Camellia sinensis*; *O*-methyltransferase; *O*-methylated EGCGs; DPPH; antibacterial; histamine release

INTRODUCTION

Tea is one of the most commonly consumed beverages in the world. Tea catechins are major polyphenols and have been studied for their inhibitory effects on carcinogenesis (*1*), antimutagenic effects (*2*), antibacterial activity (*3*), antiviral action (*4*), free-radical-scavenging activity (*5*), anticaries action (*6*), and antiallergic action (*7*), et al. Recently, the number of patients with allergies has been rapidly increasing due to changes in the environment and the effects of airborne pollen and diesel particles. (−)-Epigallocatechin-3-*O*-gallate (EGCG) is the major component of tea catechins and is known to have strong antiallergic effects (*8*, *9*).

Various tea extracts have been assessed for their ability to inhibit the release of histamine from mouse mast cells, and "Benifuuki" cultivars were found to have a strong effect. Then, new catechins, (−)-epigallocatechin-3-*O*-(3-*O*-methyl)-gallate (EGCG3″Me) and (−)-epigallocatechin-3-*O*-(4-*O*-methyl)-gallate (EGCG4″Me) were isolated (*10*). The *O*-methylated EGCGs exhibited strong antiallergic properties against type I allergies in ovalbumine (OVA), adjuvant, and passive cutaneous anaphylaxis (PCA) tests (*10*, *11*) and against type IV allergies induced by oxazolone (*12*). Furthermore, they inhibited the IgE/Ag-induced activation of mouse mast cells, the release of histamine and leukotoriene, and the production and secretion of cytokines (*11*). In another paper, EGCG3″Me reportedly inhibited degranulation by human basophilic cells (*13*). These reports indicated that *O*-methylated EGCGs have stronger antiallergic effects than EGCG.

To study the *O*-methylated EGCGs, we isolated the gene for an *O*-methyltransferase that catalyzes the methylation of EGCG from tea. Then, a number of *O*-methylated EGCGs were synthesized using this *O*-methyltransferase. Here, we studied the relationship between the number and position of methyl groups and their effects on histamine release. Moreover, other major effects of EGCG such as 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity and antibacterial activity were identified.

*To whom correspondence should be addressed. Phone: +81-297-46-1504. Fax: +81-297-46-1506. E-mail: masanobu.kirita@asahibeer.co.jp.

 Published on Web 05/17/2010

Figure 1. The deduced amino acid sequence of CsOMT was compared with those of other Caffeoyl-CoA *O*-methyltransferases (CCoAOMT) from *N. tobacum*, *P. crispum*, and *V. vinifera*. Conserved amino acids are indicated as white characters. Highly conserved motifs of the SAM binding domain, the 177−186, 196−206, and 220−229 amino acid regions, of CsOMT are proposed for plant CCoAOMT.

MATERIALS AND METHODS

Reagents. "Benifuuki" was cultivated at the National Institute of Vegetable and Tea Science, National Agriculture and Food Research Organization, Shizuoka, Japan. All chemicals and solvents were of a high or HPLC grade. Caffeoyl-CoA was synthesized according to a modification of a previous method (*14*). (−)-Epicatechin-3-*O*-gallate (ECG) was prepared from tea. EGCG3″Me and EGCG4″Me standards were prepared from "Benifuuki" tea leaves. Other HPLC standard catechins were prepared from tea or purchased from Funakoshi Co., Ltd. (Tokyo, Japan).

Isolation of the *O*-methyltransferase Gene and Expression of the Recombinant Enzyme. Total-RNA was extracted from "Benifuuki" tea leaves and used for reverse transcription-PCR (RT-PCR) to isolate the *O*-methyltrasnferase gene together with degenerated primers (forward, CAGTAYATWYTNGARACHAGTGT; reverse, TGTGRGCAACD-CCRGCTTTYTBAAT) designed with reference to the cDNA sequences of other plants. The amplified cDNA was cloned into pGEM-T (Promega Corporation, Madison, WI) and introduced into *Escherichia coli* JM109 cells to confirm its partial nucleotide sequence. To isolate a full-length cDNA fragment, specific primers were designed based on the partial cDNA nucleotide sequence. The 5′ forward primers were 5′ GSP1 (TTCGAAGT-TTTCTCTGTTAATGTC), 5′ GSP2 (GTGGCGAGAAGAGAGTAA-CAA), and 5′ GSP3 (CGGCAGAAGGTAGTCATGATGT). The 3′ reverse primer was 3′ GSP1 (GAGACCAGTGTTTACCCAAGAGAG). Using these specific primers, 5′ and 3′ RACE-PCR techniques were performed.

The full-length cDNA was digested from pGEM-T using *Nde*I and *Bam*HI sites that were introduced using specific primers (forward, TTAGCTC*CATATG*GCAACAAACGGAGAAGGAGA, italics indicate the *Nde*I site and underlining indicates the 5′ terminal sequence; reverse, TTAGCT*GGATCC*TCAGCAGACACGCCGGCA, italics indicate the *Bam*HI site and underlining indicates the 3′ terminal sequence) during PCR. The cDNA fragment was inserted into the *Nde*I and *Bam*HI sites of the pET28a (+) expression vector and transferred into *E. coli* BL21 (DE3) cells. The transformants were cultured in LB medium until an $O.D._{600}$ of 0.6 was reached. Isopropylthiogaractoside was added to the culture at a final concentration of 1 mM, and the cells were cultured at 28 °C for 5 h with shaking. Then the cultures were centrifuged to harvest the cells and suspended in 20 mM phosphate buffer (pH7.4) containing 1 mM dithiothreitol. The cell suspension was then sonicated and centrifuged again before the supernatant was used as a crude enzyme solution.

Enzyme Activity. *O*-Methyltransferase activity was measured to assess whether a methyl group had been introduced into EGCG. The 5 mL reaction mixture contained 100 mM Tris-HCl (pH7.4), 0.2 mM $MgCl_2$, 25 μM EGCG, 0.4 mM *S*-adenosyl-L-methyonine (SAM) as a methyl donor, and 1 mL of crude enzyme solution. The mixture was incubated at 30 °C for 1 h, and then the reaction was stopped with the addition of 1N HCl. The reaction mixture was extracted with 8 mL of ethyl acetate, and the organic phase was collected by centrifugation. The organic phase was dried under N_2 gas and resuspended in a 1% ascorbic acid solution. The enzyme activity was analyzed by HPLC coupled with a UV detector (Shimadzu Corporation, Kyoto, Japan). HPLC was performed with a reversed-phase Wakopak navi C-18 column (4.6 mm i.d. × 150 mm; particle size, 5 μm; Wako Pure Chemical Industries, Ltd., Osaka, Japan) at 40 °C. The flow rate was 1 mL/min. The elution was performed with 80% mobile phase A (H_2O:acetonitrile:H_3PO_4 = 400:10:1) and 20% mobile phase B (mobile phase A:methanol = 2:1) for 2 min, followed by a reduction from 80% to 20% mobile phase A for 25 min, then 20% mobile phase A for 18 min, and finally a return to 80% A for 10 min.

Determination of *O*-methylated EGCGs. High resolution time of flight mass spectrometry (HR-TOF-MS) was performed on a QSTAR Elite (Applied Biosystems, Inc., CA) equipped with an ESI source. The data acquisition was under the control of Analyst QS 2.0 software. The TurboIonSpray source was set to 450 °C, and the instrument was scanning in the range m/z 100−1000. The optimized instrument settings were as follows: ion spray voltage, −4500 V; auxiliary gas, 50 psi; nebulizer gas, 50 psi; nitrogen curtain gas, 30 psi; declustering potential, −30 V; focusing potential, −250 V; declustering potential 2, −15 V; accumulation time, 1 s. UV spectra were obtained using a HITACHI U-2000 spectrophotometer (Hitachi, Ltd., Tokyo, Japan). ^1H NMR (600 MHz), ^{13}C NMR (150 MHz), HSQC, and HMBC spectra were recorded in CD_3OD using a Bruker AV600 instrument (Bruker BioSpin GmbH, Germany). The isolation and purification of compounds were carried out with a Hitachi HPLC system, a L-7150 pump, a L-7420 UV−vis detector, and a D-2500 chromato-integrator (Hitachi, Ltd.). The reaction mixture was scaled up, as described in Materials and Methods. The *O*-metylated EGCGs were fractionated by HPLC, with a reversed-phase Inertsil ODS-3 column (20 mm i.d. × 250 mm, GL Sciences Inc., Tokyo, Japan). The detection wavelength was 280 nm, and flow rate was 12 mL/min. Mobil phase was 35% (v/v) MeOH/H_2O. The fraction were evaporated and lyophilized to give *O*-methylated EGCGs.

DPPH Radical Scavenging Assay. The DPPH radical scavenging abilities of EGCG and the other *O*-methylated EGCGs were measured. One hundred μL of 750 μM DPPH in methanol were added to each well of a 96-well plate followed by 100 μL of samples. The mixture was incubated at room temperature for 30 min, and absorbance was measured at 517 nm. The concentration causing 50% inhibition of DPPH (IC_{50}) was calculated.

Antibacterial Activity. The antibacterial activities of EGCG and *O*-methylated EGCGs were evaluated by assessing their minimum inhibitory concentrations (MIC in all cases) against *Staphylococcus aureus* (NBRC12732), *Escherichia coli* (NBCR3972), *Salmonella enterica* subsp. *enterica* (NBCR3313), *Pseudomonas aeruginosa* (NBRC13275), and *Legionera pneumophila* (ATCC33153). After overnight preculture with Mueller Hinton II agar (MH II, Becton, Dickinson and Company,

NJ) in all cases at 37 °C, the bacteria were transferred to Mueller Hinton Broth (MH Broth, Becton, Dickinson and Company, NJ) and cultured at 35 °C for 20 h. Then, 1×10^6 colony forming units (CFUs)/mL of the bacterial cells were prepared with MH Broth. *L. pneumophila* was inoculated into BCYEα agar medium (Nikken Bio Medical Laboratory, Kyoto, Japan) and incubated at 35 °C for 3 days, and 1×10^6 CFUs/mL of *L. pneumophila* bacterial cells were prepared with distilled water. The test samples were dissolved in dimethyl sulfoxide. One hundred μL of each sample were mixed with MH II ager for *S. aureus*, *E. coli*, *S. enterica* subsp. *enterica*, and *P. aeruginosa*, or *Legionella* agar (Becton, Dickinson Co., NJ) for *L. pneumophila*. The final concentrations of each test sample were 0.03, 0.063, 0.0125, 0.25, 0.75, and 1.5 mg/mL, respectively. Then, the MIC were determined after incubation at 35 °C for 20 h.

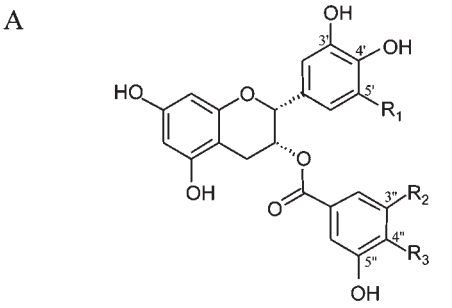
Measurement of Histamine Release. Bone marrow cells from the femurs of NC/Nga mice (Charles Liver, Kanagawa, Japan) were cultured in 4 ng/mL of murine recombinant IL-3-containing RPMI1640 medium supplemented with 10% heat-inactivated fetal bovine serum (IL-3: Peprotec, London, UK, NJ; medium: Invitrogen Lfe Technologies, Carlsbad, CA), 2 mM glutamine, and 50 μM 2-mercaptoethanol in humidified 95% air 5% CO_2 at 37 °C. More than 95% pure mast cells were obtained as bone marrow-derived mast cells (BMMC in all cases) after four weeks of culture. The BMMCs were passively sensitized at a density of 2×10^6 cells/mL with 1 μg/mL of antitrinitrophenyl (TNP) mouse monoclonal IgE antibody (BD Pharmingen, San Diego, CA) at 37 °C overnight. After being washed in Tyrode buffer (Ca^{2+}-free; 10 mM HEPES, pH 7.4, Wako Chemical; containing 0.8% NaCl, 0.02% KCl, 0.056% NaH_2PO_4, 0.1% glucose, 0.05% gelatin, and 1 μM $MgCl_2/6H_2O$), the cells were resuspended in Tyrode buffer at a density of 1×10^7 cells/mL, incubated for 20 min with EGCG and *O*-methylted EGCGs at 37 °C, and then stimulated with 300 ng/mL of TNP-BSA (TNP bovine serum albumin; Cosmo Bio Corporation, Ltd., Tokyo, Japan) and 300 μM $CaCl_2$ for 10 min at 37 °C. The reactants were added to 4 mM EDTA/Tyrode solution and cooled on ice to stop the reaction. To measure histamine, the solutions were centrifuged, an equivalent volume of 0.1N HCl was added to the supernatant, and the released histamine was measured by on-column HPLC (*15*). The HPLC system was coupled with a fluorescent photometric detector (ex. 330 nm, em. 430 nm) (Shimadzu Corporation, Kyoto, Japan) and a reverse-phase Asahipak-ODP-50-4E column (4.6 mm mm i.d. × 250 mm; particle size, 5 μm) (Showa Denko K.K., Tokyo, Japan). The solution was eluted with 50 mM sodium borate−acetonitrile (80:20) buffer containing 1 mM *o*-phthalaldehyde and 1 mM *N*-acetylcysteine at a flow rate of 0.5 mL/min at 37 °C.

RESULTS

Isolation of the *O*-Methyltransferase Gene and Expression of the Recombinant Enzyme. The identified cDNA fragment (*CsOMT*) was composed of a 735-nucleotide open reading frame (ORF) encoding 245 amino acids (**Figure 1**). A database search revealed that the deduced amino acid sequence of CsOMT had more than 89% identity with the Caffeoyl-CoA *O*-methyltransferases (CCoAOMT) of *Vitis vinifera*, *Petroselinum crispum*, and *Nicotiana tobacum* (*16*−*18*). The amino acid sequence included highly conserved motifs A, B, and C, which are probably SAM-binding motifs (*19*).

The recombinant enzyme was analyzed by 12% SDS-PAGE, and an expressed band was clearly recognized (data not shown). The molecular weight of CsOMT was estimated to be approximately 27.6 kDa based on 12% SDS-PAGE and its amino acid sequence.

CsOMT Activity and Determination of New *O*-Methylated EGCG Derivatives. Using the crude recombinant enzyme, *O*-methyltransferase activity was measured and the reaction mixture was analyzed by HPLC. As a result, EGCG3″Me and EGCG4″Me peaks were detected at the same retention times as found for the standards for *O*-methylated EGCGs (**Figure 2**). EGCG3″Me and EGCG4″Me were measured using LC/MS. The molecular weight of EGCG3″Me was analyzed and found to be 471.4 ([M − H]$^-$), which was consistent with its theoretical value. EGCG4″Me was



Compounds	R_1	R_2	R_3
(−)-epicatechin-3-*O*-gallate (ECG)	H	OH	OH
(−)-epigallocatechin-3-*O*-gallate (EGCG)	OH	OH	OH
(−)-epigallocatechin-3-*O*-(3-*O*-methyl)-gallate (EGCG3"Me)	OH	OCH_3	OH
(−)-epigallocatechin-3-*O*- (4-*O*-methyl)-gallate (EGCG4"Me)	OH	OH	OCH_3

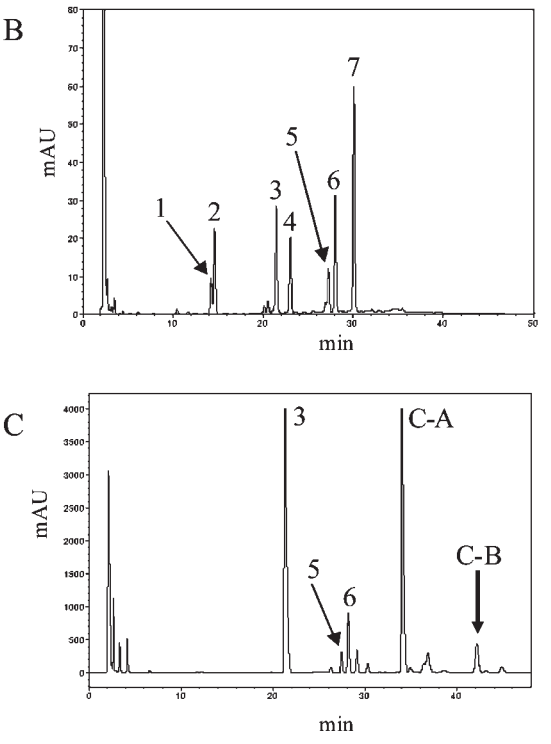
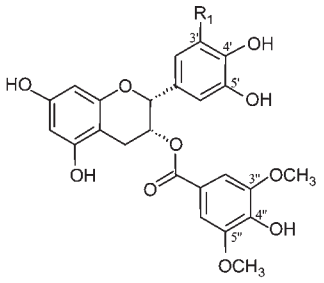


Figure 2. HPLC analysis of the enzymatic reaction products using CsOMT. UV detection at 272 nm. (A) Chemical structures of ECG, EGCG, and *O*-methylated EGCGs. (B) Standard catechins. Peak identification: 1, EGC; 2, (−)-catechin; 3, EGCG; 4, EC; 5, EGCG4″Me; 6, EGCG3″Me; 7, ECG. (C) Enzymatic reaction products. Enzymatic activity was analyzed by HPLC. Two major unknown compounds, C−A and C−B, were detected.

similarly analyzed and confirmed. However, two major unknown peaks were detected in the HPLC chromatogram (Figure**2**). To confirm these unknown peaks, the compounds were fractionated.

Compound-A was obtained as a white amorphous powder. Its molecular formula was deduced to be $C_{24}H_{22}O_{11}$ on the basis of the negative HR-TOF-MS (*m*/*z* 485.1094 [M − H]$^-$, calcd 485.1078), together with the ^{13}C NMR spectral data. The ^1H NMR spectrum of compound-A was similar to that of EGCG (*20*), except for the presence of a methoxyl proton signal at δ 3.82 (6H, s), and showed two methine proton signals at δ 5.04 (H-2, 1H, br s) and δ 5.47 (H-3, 1H, m), two methylene proton signals at

δ 2.92 (H-4, 1H, dd, J = 17.4, 3.6 Hz) and δ 3.00 (H-4, 1H, dd, J = 17.4, 4.8 Hz), and four aromatic proton signals at δ 5.96 (H-8, 1H, d, J = 2.4 Hz), δ 5.98 (H-6, 1H, d, J = 2.4 Hz), δ 6.53 (H-2′, H-6′, 2H, s) and δ 7.15 (H-2″, H-6″, 2H, s). The aromatic region of the



Compounds	R_1
C-A	
(−)-epigallocatechin-3-*O*-(3,5-di*O*-methyl)-gallate (EGCG3",5"diMe)	OH
C-B	
(−)-3-*O*-methyl-epigallocatechin-3-*O*- (3,5-*O*-dimethyl)-gallate (EGCG3 ',3",5"triMe)	OCH_3

Figure 3. Chemical structures of unknown compound-A (C−A) and -B (C−B), which were synthesized in an enzymatic reaction using CsOMT. Their chemical structures were established by negative HR-TOF-MS and NMR.

Table 1. ^{13}C and ^{1}H NMR Spectra Data for Compound-A and Compound-B Measured in CD_3OD (150 MHz for ^{13}C, 600 MHz for ^{1}H)[a]

position	compound-A		position	compound-B	
	δC	δH		δC	δH
2	78.4	5.04 (br s)	2	78.8	5.07 (br s)
3	70.8	5.47 (m)	3	70.6	5.55 (m)
4	26.3	2.92 (dd = 3.6, 17.4)	4	26.7	2.92 (dd = 3.0, 17.4)
		3.00 (dd = 4.8, 17.4)			3.03 (dd = 4.8, 17.4)
5	157.8[a]		5	157.9[b]	
6	96.3	5.98 (d = 2.4)	6	95.7	6.00 (d = 2.4)
7	158.0[a]		7	158.1[b]	
8	96.5	5.96 (d = 2.4)	8	96.6	5.98 (d = 2.4)
9	157.1		9	157.3	
10	99.3		10	99.4	
1′	131.0		1′	130.7	
2′, 6′	106.7	6.53 (s)	2′	103.2	6.58 (d = 1.5)
3′, 5′	146.9		3′	149.4	
4′	133.7		4′	134.9	
1″	121.6		5′	146.4	
2″, 6″	108.2	7.15 (s)	6′	108.7	6.65 (d = 1.5)
3″, 5″	148.8		OMe	56.5	3.62 (s)
4″	141.8		1″	121.5	
OMe	56.8	3.82 (s)	2″, 6″	108.3	7.17 (s)
C═O	167.6		3″, 5″	148.9	
			4″	142.2	
			OMe	56.8	3.81 (s)
			C═O	167.3	

[a] a b Each signal was interchangeable on the same column.

^{13}C NMR spectrum showed the presence of six carbon signals at δ 121.6 (C-1″), 108.2 (C-2″, C-6″), 148.8 (C-3″, C-5″), and 141.8 (C-4″), characteristic of a galloyl group, suggesting a symmetrical structure. In the HMBC spectrum, the methoxyl proton signal at δ 3.82 was correlated to C-3″ (C-5″) and H-2″ (H-6″) were correlated to C-1″, C-2″ (C-6″), C-3″ (C-5″) and an ester carbonyl carbon (C═O). These data indicated that two methoxy groups were located at C-3″ and C-5″ of the galloyl moiety. Therefore, compound-A was established as (−)-epigallocatechin 3-*O*-(3, 5-*O*-dimethyl)-gallate (EGCG3″, 5″diMe) (**Figure 3**, **Table 1**).

Compound-B was obtained as a white amorphous powder. Its molecular formula was deduced to be $C_{25}H_{24}O_{11}$ on the basis of the negative HR-TOF-MS (m/z 499.1242 $[M - H]^-$, calcd 499.1234) together with the ^{13}C NMR spectral data. This data indicated three methoxy groups in EGCG. The ^{1}H NMR spectrum of compound-B was similar to that of EGCG3″,5″diMe, except for the presence of another methoxyl proton signal at δ 3.62 (3H, s) and two aromatic proton signals at δ 6.58 (H-2′, 1H, d, J = 1.5 Hz), δ 6.65 (H-6′, 1H, d, J = 1.5 Hz) while the signal of δ 6.53 (H-2′, H-6′, 2H, s) in EGCG3″, 5″diMe. In the HMBC spectrum, the methoxyl proton signal at δ 3.62 was correlated to C-3′ and H-2′ correlated to C-1′, C-3′, C-4′, C-6′, and C-2 while H-6′ correlated to C-2′, C-4′, C-5′, and C-2. These data indicated that another methoxy group was located at the C-3′ of the B ring. Therefore, compound-B was established as (−)-3-*O*-methyl-epigallocatechin-3-*O*-(3, 5-*O*-dimethyl)-gallate (EGCG3′, 3″, 5″triMe) (**Figure 3**, **Table 1**).

DPPH Radical Scavenging Assay. DPPH radical scavenging activity of each compound was measured, and their IC_{50} values were calculated. The IC_{50} value for EGCG was 14.7 μM, that for ECG was 20.1 μM, that for EGCG3″Me was 25.1 μM, that for EGCG4″Me was 42.7 μM, that for EGCG3″,5″diMe was 36.1 μM, and that for EGCG3′,3″,5″triMe was 50.7 μM; i.e., EGCG was shown to have the strongest action.

Antibacterial Activity. The MICs of EGCG and *O*-methylated EGCGs were evaluated for their activity against five bacterial strains (**Table 2**). EGCG4″Me was excluded from this study because only a small amount of it was produced. EGCG was shown to be the most effective at inhibiting the bacterial strains, and in particular, it demonstrated a strong effect against *S. aureus*. EGCG3″,5″diMe had no effect on *E. coli*, and EGCG3′,3″,5″-triMe had no effect on *E. coli* or *S. enterica* subsp. *enterica* even at a high concentration in this study.

Inhibition of Histamine Release. The antiallergic effects of EGCG and *O*-methylated EGCGs were measured in terms of the release of histamine from mouse mast cells. EGCG4″Me was excluded from this study because only a small amount of it was produced. The order of ability to inhibit histamine release was EGCG3′,3″,5″triMe = EGCG3″,5″diMe > EGCG3″Me > EGCG (**Figure 4**).

DISCUSSION

In this study, we isolated the gene of an *O*-methyltransferase, *CsOMT*, from tea. Two *O*-methyltransferase genes have been

Table 2. Antibacterial Activity of EGCG and *O*-Methylated EGCGs

	MIC(mg/mL)			
bacteria strains	EGCG	EGCG3″Me	EGCG3″,5″diMe	EGCG3′,3″,5″triMe
Staphylococcus aureus	0.125	0.125	0.25	0.75
Escherichia coli	0.75	1.5	>1.5	>1.5
Salmonella enterica subsp. enterica	0.25	0.75	0.75	>1.5
Pseudomonas aeruginosa	0.75	0.75	0.75	>1.5
Legionella pneumophila	0.25	0.75	0.75	0.75

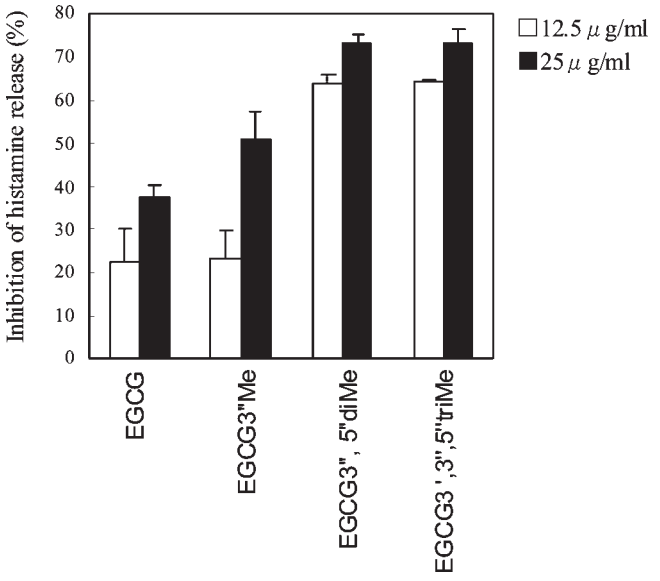


Figure 4. The effects of *O*-methylated EGCGs on histamine release from BMMC. The inhibition of histamine release was expressed as a percentage reduction compared to the control values (histamine release induced by TNP-BSA without inhibitors).

isolated previously, caffeine synthase and selenocysteine methyltransferase from tea, but their deduced amino acid sequences were 369 and 351 amino acid in length, respectively (*21*, *22*), whereas the CsOMT sequence has a different amino acid length and so belongs to another class of *O*-methyltransferase. The *O*-methyltransferases of plants are categorized into two major classes (*19*). The class I enzymes are 231−248 amino acid long, and the class II enzymes are 344−383 long. Therefore, this is first time that a class I *O*-methyltransferase has been isolated from tea. The deduced amino acid sequence of CsOMT was highly homologous with the CCoAOMT used for the biosynthesis of lignin in plants. Thereby, caffeoyl-CoA was synthesized according to a modification of a previous method (*14*) and assayed with the recombinant enzyme of CsOMT. From the LC/MS analysis, the presence of *O*-methylated Caffeoyl-CoA was confirmed (data not shown). CCoAOMT catalyzes the methylation of caffeoyl-CoA and 5-hydroxyferuloyl-CoA to feruloyl-CoA and sinapoyl-CoA, respectively (*23*, *24*). Because of the structural similarity between caffeoyl-CoA or 5-hydroxyferuloyl-CoA and EGCG, CsOMT is able to recognize galloyl moieties. CCoAOMT has significant similarity at the amino acid level with the catechol *O*-methyltransferases from rats and humans (*25*, *26*). Cytosolic catechol *O*-methyltransferase catalyzes the methylation of EGCG to EGCG4″Me or EGCG4′,4″diMe, that of EGC to EGC4′Me (*27*). According to these reports, CsOMT is able to recognize and catalyze the methylation of EGCG.

CsOMT was also able to synthesize EGCG3″,5″diMe and EGCG3′,3″,5″triMe, which have never been identified in tea. EGCG3″,5″diMe was purified from *Bryophyllum pinnatum* (Lam) Oken and *Stryphnodendron adstringens* (Martiuc) Coville, which are medical plants grown in West Africa and Brazil, respectively (*28*, *29*). However, only minor quantities are contained in these plants, and there has been no other attempt to purify or detect EGCG3″,5″diMe from other plants including tea cultivars and no reports have attempted to identify EGCG3′,3″,5″triMe.

To assess the major effects of EGCG, their DPPH radical scavenging ability and antibacterial activity were evaluated. Regarding antioxidant activity, the order of DPPH IC_{50} was EGCG > ECG > EGCG3″Me > EGCG3″,5″diMe > EGCG4″Me > EGCG3′,3″,5″triMe. These results indicated that reducing the number of hydroxyl groups decreases the DPPH radical scavenging activity, although this was not the case for EGCG4″Me. In spite of the same number of hydroxyl groups, ECG showed a more similar DPPH activity to EGCG than EGCG3″Me, so decreasing the number of hydroxyl groups in the B ring seem to have less effect than doing the same to the galloyl moiety. In spite of the partial methylation of the hydroxyl group in their galloyl moieties, EGCG3″Me and EGCG4″Me showed different levels of activity. In another report, glucosylation of 4′ in the B ring and 4″ in the galloyl moiety of EGCG seemed to decrease its DPPH radical scavenging activity, while after glycosylation of only the 4′ in the B ring left its action relatively unchanged (*30*). Thus, methylation of the 4″ position has more influence on the activity of DPPH than that of other positions in the galloyl moiety.

The trolox equivalent antioxidant capacity values of trimethylated ECG (three hydroxyl groups of its galloyl moiety are methylated) were evaluated to assess the antioxidant activity of other catechins (*31*). This report indicated that the presence of the galloyl moiety and its hydroxyl groups induced antioxidant activity, which supports our results. Regarding the MIC against the five bacterial strains, EGCG indicated the strongest activity. We predicted that increasing the number of methylated galloyl moieties would increase the stability of the molecule and help it acquire lipophilicity and thereby increase its antibacterial activity. Therefore, octanol−water partition coefficients by shaking-flask method, as the index of lipophilicity were confirmed as described method (*32*). The log *Po*/*w* value of EGCG, EGCG3″Me, EGCG3″,5″diMe, and EGCG3′,3″,5″triMe were 0.61, 1.04, 1.37, and 1.46, respectively. However, the result was same as that for DPPH activity.

The antiallergic effects of EGCG and *O*-methylated EGCGs were assessed by measuring the amount of histamine release from mouse mast cells (*11*) and the basophilic cell line KU812 (*33*). EGCG3″Me showed stronger inhibitory effects than EGCG as reported previously. On the other hand, there has been no report about the antiallergic activity of EGCG3″,5″diMe or EGCG3′,3″,5″triMe. In this study, we examined the relationship between the number of methylated galloyl moieties and inhibitory histamine release. EGCG3″,5″diMe showed stronger inhibition of histamine release than EGCG3″Me. According to these results, the methylation of the 5″ position enhanced the inhibition of histamine release. Because of the similar effect on histamine release of EGCG3″Me and EGCG4″Me (*11*), the number of the methylated galloyl moieties is considered to be more important than their position with regard to inhibiting histamine release. EGCG3′,3″,5″triMe had an approximately equal effect to EGCG3″,5″diMe. These results suggested that the methylation of the hydroxyl groups of the B ring did not contribute to histamine release inhibition, at least at the 3′ position. The EGCG receptor is a 67 kDa laminin receptor (67LR) expressed on the surface of cancer cells (*34*). After binding to the 67LR of EGCG or *O*-methylated EGCGs, downstream of biosynthesis is regulated and down-regulated the FcεRI expression, which is the high-affinity IgE receptor (*35*). The ability to bind to the 67LR of EGCG3″Me was lower than that of EGCG, in spite of it having a stronger inhibitory effect on histamine release than EGCG. Further research is required into the ability of EGCG3″,5″diMe and EGCG3′,3″,5″triMe to bind to the 67LR and other binding receptors.

In this study, we identified the relationship between the number and position of methyl groups. The reducing the number of hydroxyl groups decreased the effectiveness of DPPH and antibacterial activity. On the other hand, the inhibition of histamine release

was potentiated by an increase in the number of methyl groups. The plasma concentrations of unconjugated EGCG and EGCG3″Me were studied after the administration of a "Benifuuki" green tea beverage in healthy humans (*36*). This report indicated that EGCG3″Me is absorbed more easily than EGCG, and it was shown to be effective against cedar pollinosis. From these reports and our results, increasing the number of methyl groups of EGCG, especially in the galloyl moiety, help it to acquire lipophilicity, improves absorption and stability in animal and human plasma, and contributes to the inhibition of histamine release.

Note Added after ASAP Publication

Figure 1 caption was modified in the version of this paper published ASAP May 17, 2010; the corrected version published ASAP May 19, 2010.

LITERATURE CITED

(1) Wang, Z. Y.; Hong, J.; Huang, M.; Reuhl, K. R.; Conney, A. H.; Yang, C. S. Inhibition of *N*-nitrosodiethylamine- and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced tumorigenesis in A/J mice by green tea and black tea. *Cancer Res.* **1992**, *52*, 1943–1947.

(2) Wang, Z. Y.; Cheng, S. J.; Zhou, Z. C.; Athar, M.; Khan, W. A.; Bickers, D. R.; Mukhtar, H. Antimutagenic activity of green tea polyphenols. *Mutat. Res.* **1989**, *223*, 273–285.

(3) Fukai, K.; Ishigami, T.; Hara, Y. Antibacterial activity of tea polyphenols against phytopathogenic bacteria. *Agric. Biol. Chem.* **1991**, *55*, 1895–1897.

(4) Nakayama, M.; Suzuki, K.; Toda, M.; Okubo, S.; Hara, Y.; Shimamura, T. Inhibition of the infectivity of influenza virus by tea polyphenols. *Antiviral Res.* **1993**, *21*, 289–299.

(5) Bors, W.; Saran, M. Radical scavenging by flavonoid antioxidants. *Free Radic. Res. Commun.* **1987**, *2*, 289–294.

(6) Hattori, M.; Kusumoto, I. T.; Namba, T.; Ishigami, T.; Hara, Y. Effect of tea polyphenols on glucan synthesis by glucosyltransferase from *Streptococcus mutans*. *Chem. Pharm. Bull.* **1990**, *38*, 717–720.

(7) Kakegawa, H.; Matsumoto, H.; Endo, K. Inhibitory effects of tannins on hyaluronidase activation and on the degranulation from rat mesentery mast cells. *Chem. Pharm. Bull.* **1985**, *33*, 5079–5082.

(8) Matsuo, N.; Yamada, K.; Shoji, K.; Mori, M.; Sugano, M. Effect of tea polyphenols on histamine release from rat basophilic leukemia (RBL-2H3) cells: the structure−inhibitory activity relationship. *Allergy Eur. J. Allergy Clin. Immunol.* **1997**, *52*, 58–64.

(9) Ohmori, Y.; Ito, M.; Kishi, M.; Mizutani, H.; Katada, T.; Konishi, H. Antiallergic constituents from oolong tea stem. *Biol. Pharm. Bull.* **1995**, *18*, 683–686.

(10) Sano, M.; Suzuki, M.; Miyase, T.; Yoshino, K.; Maeda-Yamamoto, M. Novel antiallergic catechin derivatives isolated from oolong tea. *J. Agric. Food Chem.* **1999**, *47*, 1906–1910.

(11) Maeda-Yamamoto, M.; Inagaki, N.; Kitaura, J.; Chikumoto, T.; Kawahara, H.; Kawakami, Y.; Sano, M.; Miyase, T.; Tachibana, H.; Nagai, H.; Kawakami, T. *O*-Methylated catechins from tea leaves inhibit multiple protein kinases in mast cells. *J. Immunol.* **2004**, *172*, 4486–4492.

(12) Suzuki, M.; Yoshino, K.; Maeda-Yamamoto, M.; Miyase, T.; Sano, M. Inhibitory effects of tea catechins and *O*-methylated derivatives of (−)-epigallocatechin-3-*O*-gallate on mouse type IV allergy. *J. Agric. Food Chem.* **2000**, *48*, 5649–5653.

(13) Tachibana, H.; Sunada, Y.; Miyase, T.; Sano, M.; Maeda-Yamamoto, M.; Yamada, K. Identification of a methylated tea catechin as an inhibitor of degranulation in human basophilic KU812 cells. *Biosci. Biotechnol. Biochem.* **2000**, *64*, 452–454.

(14) Stoeckigt, J.; Zenk, M. H. Chemical syntheses and properties of hydroxycinnamoyl coenzyme A derivatives. *Z. Naturforsch., C: J. Biosci.* **1975**, *30*, 352–358.

(15) Saito, K.; Horie, M.; Nose, N.; Nakagomi, K.; Nakazawa, H. High-performance liquid chromatography of histamine and 1-methylhistamine with on-column fluorescence derivatization. *J. Chromatogr.* **1992**, *595*, 163–168.

(16) Martz, F.; Maury, S.; Pinçon, G.; Legrand, M. cDNA cloning, substrate specificity and expression study of tobacco caffeoyl-CoA 3-*O*-methyltransferase, a lignin biosynthetic enzyme. *Plant. Mol. Biol.* **1998**, *36*, 427–437.

(17) Busam, G.; Junghanns, K. T.; Kneusel, R. E.; Kassemeyer, H.; Matern, U. Characterization and expression of caffeoyl-coenzyme a 3-*O*-methyltransferase proposed for the induced resistance response of *Vitis vinifera* L. *Plant Physiol.* **1997**, *115*, 1039–1048.

(18) Schmitt, D.; Pakusch, A.-E.; Matern, U. Molecular cloning, induction, and taxonomic distribution of Caffeoyl-CoA 3-*O*-methyltransferase, an enzyme involved in disease resistance. *J. Biol. Chem.* **1991**, *266*, 17416–17423.

(19) Joshi, C. P.; Chiang, V. L. Conserved sequence motifs in plant *S*-adenosyl-L-methionine-dependent methyltransferases. *Plant. Mol. Biol.* **1998**, *37*, 663–674.

(20) Davis, A. L.; Cai, Y.; Davies, A. P.; Lewis, J. R. ^{1}H and ^{13}C NMR assignments of some green tea polyphenols. *Magn. Reson. Chem.* **1996**, *34*, 887–890.

(21) Kato, M.; Mizuno, K.; Crozier, A.; Fujimura, T.; Ashihara, H. Caffeine synthase gene from tea leaves. *Nature* **2000**, *406*, 956–957.

(22) Zhu, L.; Jiang, C.-J.; Deng, W.-W.; Gao, X.; Wang, R.-J.; Wan, X.-C. Cloning and expression of selenocysteine methyltransferase cDNA from Camellia sinensis. *Acta Physiol. Plant.* **2008**, *30*, 167–174.

(23) Zhong, R.; Herbert Morrison, W., III; Negrel, J.; Ye, Z.-H. Dual methylation pathways in lignin biosynthesis. *Plant Cell* **1998**, *10*, 2033–2045.

(24) Lee, D.; Meyer, K.; Chapple, G.; Douglas, C. J. Antisense suppression of 4-coumarate:coenzyme A ligase activity in *Arabidopsis* leads to altered lignin subunit composition. *Plant Cell* **1997**, *9*, 1985–1998.

(25) Salminen, M.; Lundstrom, K.; Tilgmann, C.; Savolainen, R.; Kalkkinnen, N.; Ulmanen, I. Molecular cloning and characterization of rat liver catechol-*O*-methyltransferease. *Gene* **1990**, *93*, 241–247.

(26) Bertocci, B.; Miggiano, V.; Da Prada, M.; Dembic, Z.; Lahm, H.-W.; Malherbe, P. Human catechol-*O*-methyltransferase: cloning and expression of the membrane-associated form. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 1416–1420.

(27) Lu, H.; Meng, X.; Yang, C. S. Enzymology of methylation of tea catechins and inhibition of catechol-*O*-methyltransferase by (−)-epigallocatechin gallate. *Drug Metab. Dispos.* **2003**, *31*, 572–579.

(28) Okushio, K.; Suzuki, M.; Matsumoto, N.; Nanjo, F.; Hara, Y. Methylation of tea catechins by rat liver homogenates. *Biosci. Biotechnol. Biochem.* **1999**, *63*, 430–432.

(29) De Mello, J. P.; Petereit, F.; Nahrstedt, A. Flavan-3-ols and prodelphinidins from *Stryphnodendron adstringens*. *Phytochemistry* **1996**, *41*, 807–813.

(30) Nanjo, F.; Goto, K.; Seto, R.; Suzuki, M.; Sakai, M.; Hara, Y. Scavenging effects of tea catechins and their derivatives on 1,1- diphenyl-2-picrylhydrazyl radical. *Free Radical Biol. Med.* **1996**, *21*, 895–902.

(31) Sánchez-del-Campo, L.; Otón, F.; Tárraga, A.; Cabezas-Herrera, J.; Chazarra, S.; Rodríguez-López, J. N. Synthesis and biological activity of a 3,4,5-trimethoxybenzoyl ester analogue of epicatechin-3-gallate. *J. Med. Chem.* **2008**, *51*, 2018–2026.

(32) Shibusawa, Y.; Shoji, A.; Yanagida, A.; Shindo, H.; Tagashira, M.; Ikeda, M.; Ho, Y. Determination of log $P_{o/w}$ for catechins and their isomers, oligomers, and other organic compounds by stationary phase controlled high speed countercurrent chromatography. *J. Liq. Chromatogr. Relat. Technol.* **2005**, *28*, 2819–2834.

(33) Fujimura, Y.; Tachibana, H.; Maeda-Yamamoto, M.; Miyase, T.; Sano, M.; Yamada, K. Antiallergic tea catechin, (−)-epigallocatechin-3-*O*-(3-*O*-methyl)-gallate, suppresses FcεRI expression in human basophilic KU812 cells. *J. Agric. Food Chem.* **2002**, *50*, 5729–5734.

(34) Tachibana, H.; Koga, K.; Fujimura, Y.; Yamada, K. A receptor for green tea polyphenol EGCG. *Nat. Struct. Mol. Biol.* **2004**, *11*, 380–381.

(35) Yano, S.; Fujimura, Y.; Umeda, D.; Miyase, T.; Yamada, K.; Tachibana, H. Relationship between the biological activities of methylated derivatives of (−)-epigallocatechin-3-*O*-gallate (EGCG) and their cell surface binding activities. *J. Agric. Food Chem.* **2007**, *55*, 7144–7148.

(36) Maeda-Yamamoto, M.; Ema, k.; Shibuichi, T. In vitro and in vivo anti-allergic effect of "Benifuuki" green tea containing *O*-methylated catechin and ginger extract enhancement. *Cytotechnology* **2007**, *55*, 135–142.

Received for review February 15, 2010. Revised manuscript received April 30, 2010. Accepted May 10, 2010.