JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Deodorization with Ku-ding-cha Containing a Large Amount of Caffeoyl Quinic Acid Derivatives

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Caffeoyl quinic acid (CQA) derivatives in ku-ding-cha, mate, coffee, and related plants were determined by HPLC. One ku-ding-cha contained a large amount of 3,5-dicaffeoylquinic acid (3,5-diCQA, 10.6% in dry weight) as well as 3-CQA (1.7%), 4-CQA (1.1%), 5-CQA (6.3%), 3,4-diCQA (1.8%), and 4,5-diCQA (4.3%). In this ku-ding-cha, the total caffeic acid moiety was 90.3 mmol/100 g of dry weight. The leaves of *Ilex latifolia*, which is one original species of ku-ding-cha, and another plant of the same genus, *I. rotunda*, also contained 3,5-diCQA (9.5 and 14.6%), 3-CQA (4.3 and 1.9%), and 5-CQA (4.8 and 3.8%), respectively, whereas raw coffee bean contained 5.5% 5-CQA and other low CQA derivatives. 3,5-DiCQA and 5-CQA with an apple acetone powder (AP) containing polyphenol oxidase showed high capturing activities toward thiols, and two addition compounds between 3,5-diCQA and methane thiol were also identified. Ku-ding-cha indicated extremely strong capturing activities toward methanethiol, propanethiol, and 2-propenethiol in the presence of apple AP. Furthermore, drinking ku-ding-cha reduced the amount of allyl methyl sulfide gas, well-known to persist as malodorous breath long after the ingestion of garlic.

KEYWORDS: Enzymatic deodorization; bad breath; ku-ding-cha; *Ilex latifolia*; *Ilex rotunda*; acetone powder; caffeoylquinic acid; thiol; allyl methyl sulfide

INTRODUCTION

Ku-ding-cha is generally consumed in southern China as a tea-like beverage. The color of the beverage is light green, but the taste is very bitter. This nature is the origin of the Chinese name, ku-ding-cha (*1*). The bitter taste is known to be caused by terpenes and their glycosides (*2*, *3*). It has been reported that triterpenoids from ku-ding-cha have inhibitory effects on acyl CoA cholesteryl acyl transferase (*4*, *5*). These compounds may serve as new types of medicines to treat arteriosclerosis and obesity (*4*). When we examined ku-ding-cha extracts by two-dimensional paper chromatography, two spots emitting blue-white fluorescence were detected. These spots are likely chlorogenic acid and isochlorogenic acid (3,5-dicaffeoylquinic acid). The coffee bean is well-known as a plant containing caffeoylquinic acid (CQA) derivatives (*6*, *7*). Mate, a traditional beverage in South America, has also been reported to contain these compounds (*8*).

The original plants of ku-ding-cha consist of about 10 known species, *Ligustrum pedunclare*, *Ligustrum purpurascens*, *Ligustrum japonicum* var. *pubescens*, *Ligustrum robustrum*, *Ilex cornuta*, *Ilex kudincha*, *Ilex latifolia*, *Cratoxylum prunifolium*, *Ehretia thyrsiflora*, and *Photinia serruiata* (*1*, *9*). The main plants are *I. cornuta*, *I. kudincha*, and *I. latifolia* (*10*), which belong to the same genus as mate (*Ilex paraguariensis*). In ancient times, leaves of *I. latifolia* were used as paper or cards for writing brief letters in temples, because black letters remained on the leaves after writing injured the surface of the leaves. This phenomenon has been thought to be caused by the reactions of large amounts of polyphenolic compounds and polyphenol oxidases in the leaves.

Recently we have been studying enzymatic deodorization, a novel method involving the use of polyphenolic compounds (PPs) and polyphenol oxidases (PPOs) or peroxidases (PODs) to remove bad odors from the mouth and the environment (*11*−*16*). We have previously demonstrated that deodorization with foods is achieved by multiple actions including physical and chemical interactions between volatile sulfur compounds from *Allium* species and foods, enzymatic degradation of disulfides, and addition of thiols to PPs catalyzed by PPOs or PODs (*16*).

On the other hand, the formation of volatile sulfur compounds from *Allium* species (*17*) and *Allium* breath volatiles (*18*−*22*) have been investigated in detail. It is well-known that malodorous breath (halitosis) can originate in the mouth as well as in

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10.1021/jf049693j CCC: $27.50
Published on Web 07/17/2004

the gut, particularly in the case of sulfur compounds produced after the ingestion of *Allium* species. Furthermore, it has been demonstrated that most allyl methyl sulfide (AMS) gas originates from the gut (rather than the mouth), and this gas is likely to account for the well-known persistence of malodorous breath long after garlic ingestion (*19*). AMS is presumed to form via the methylation of 2-propenethiol (*22*) or the methylation of diallyl disulfide and diallyl sulfide followed by the breakdown of the disulfide and sulfide−carbon bond (*23*).

These studies suggest that ku-ding-cha containing CQA derivatives has great potential for deodorization. The purpose of this study was to determine CQA derivatives in ku-ding-cha and related plants and to investigate the effect of ku-ding-cha on the in vivo removal of AMS as well as thiols.

MATERIALS AND METHODS

Tea Products and Plants. Several ku-ding-cha varieties were purchased from tea stores in Yunnan and Shanghai, China, and coffee beans (*Coffea arabica*), mate (*I. paraguariensis*, product made in Argentina), and teas (green tea, black tea, and oolong tea) from supermarkets in Japan. Fresh leaves of *Ilex* species *I. latifolia*, *I. integra*, *I. rotunda*, *I. macropoda*, and *I. cornuta* were collected from the botanical gardens of Tsukuba University and Kagawa Nutrition University. The leaves were lyophilized and stored at −20 °C.

Chemicals. A 15% sodium methanethiolate aqueous solution, propanethiol, 2-propenethiol, AMS, dimethyl sulfide, dimethyl disulfide, diallyl sulfide, diallyl disulfide, and chlorogenic acid were purchased from Tokyo Chemical Industry Co., Ltd., Tokyo, Japan. Wakosil 25C18 was obtained from Wako Pure Chemical Industry, Osaka, Japan. Detector tubes (no. 70L), which were used for measuring the amount of thiols, were products of Gastec Corp., Kanagawa, Japan (*12*, *15*). Silica gel adsorbed $HgCl_2$ was packed in the detector tubes (5 × 130 mm) and thiol gas passed through the tube. The following reaction occurred: $RSH + HgCl_2 \rightarrow RSHgCl + HCl$. The yellow color of cresol red in the tube as a pH indicator changed to red with HCl as a reaction product.

Isolation of 3,5-Dicaffeoylquinic Acid (3,5-DiCQA). The powder (20 g) of ku-ding-cha was extracted with 80% MeOH (200 mL) by stirring for 5 h. After the solution was filtered through a filter paper under suction, the residue was re-extracted twice with the same solvent (200 mL) for 5 h. The solution was filtered, and the combined filtrates were concentrated to ∼100 mL in a rotary vacuum evaporator at 45 °C. One hundred milliliters of water was added to the concentrate, and the solution was extracted six times with 100 mL of chloroform to remove chlorophyll. After the removal of chloroform from the solution, the pH was adjusted to ∼4 with 2 N HCl. The solution was extracted seven times with 100 mL of ethyl acetate. The ethyl acetate layer was evaporated to dryness under reduced pressure and further dried in a desiccator with a vacuum to yield 1.58 g. A portion of the dry matter (0.50 g) was dissolved in 20 mL of MeOH/1% AcOH (20:80) and loaded onto a Wakosil 25C18 column (2.5 × 32 cm) equilibrated with the same solvent. After the column had been washed with the same solvent, a stepwise elution with MeOH concentrations of 30, 40, 50, 60, and 80% in the solvent system of MeOH/1% AcOH was performed. Eluates were monitored by HPLC and identified by LC-MS. 3,5-DiCQA was eluted with 40% MeOH with 1% AcOH. The fractions containing 3,5-diCQA were combined, concentrated in a rotary vacuum evaporator, and lyophilized to yield 0.20 g of powder (crude 3,5-diCQA). Crude 3,5-diCQA (86.1 mg) was dissolved in absolute ethyl acetate and chloroform added to precipitate (*24*) 66.6 mg of a white 3,5-diCQA powder. The ^1H NMR spectrum of 3,5-diCQA closely coincided with those by Noguchi et al. and Chuda et al. (*25*−*27*). Furthermore, $[M + H]^+$ at *m*/*z* 517, $[M + Na]^+$ at *m*/*z* 539, and $[(M + H) - H_2O]^+$ at *m*/*z* 499 were detected by electrospray ionization (ESI) mass spectrum.

Preparation of the Acetone Powder (AP). Raw unripe apples (cv. Ourin) or Japanese pears (cv. Housui) (100 g) were homogenized with 400 mL of cold acetone (−20 °C) in a Waring blender. The homogenate was filtered through a filter paper under suction and the residue further homogenized with 400 mL of 80% acetone. After filtration, the residue was washed twice with 80% acetone (200 mL) at 4 °C, acetone removed, and the residue lyophilized. The acetone powders containing enzymes such as PPOs and PODs was stored at −20 °C. It was reported that apple AP (or PPO) and pear AP (or PPO) were more specific for chlorogenic acid (5-CQA) and (−)-epicatechin (EC) (*11*), respectively.

Assay of PPO Actvity. PPO activity was assayed by direct colorimetric method (*28*). The mixtures of 2 mg of AP and 2 mL of 0.1 M potassium phosphate buffer (pH 6.0) were well suspended and preincubated at 30 °C in each test tube, and then the reactions were initiated by the addition of 2 mL of 10 mM 5-CQA or EC solution. After the incubations for 1, 2, and 4 min, the reactions were stopped by the additions of 0.2 mL 2 N HCl. Furthermore, after centrifugation at 3500 rpm for 5 min, absorbances of the supernatants at 420 nm were measured. One unit of activity was defined as the amount of enzyme that induced a change of 0.01/min in absorbance.

Measurements of the Thiols and AMS-Capturing Activities. *(i) Reactions with Thiols.* To a mixture of 10 mg of apple AP (or 20 mg of pear AP) and 100 μL of a 0.1% thiol aqueous suspension in a 30-mL borosilicate glass vial with open-top screw cap and Teflon/silicon disk (Pierce) was added 2.0 mL of a beverage that was extracted from 1.0 g of tea powder with 50 mL of hot water. The vial was shaken by hand at a rate of 2 strokes/s at 25 °C for 3 min. An aliquot volume of the headspace gas (2−6 mL) was passed through a detector tube. The effect of acetone powder and CQA was measured by mixing 2 mg of apple AP, 1.0 mL of a 0.1 M acetate buffer (pH 5.0), 0.8 mL of a 2.5 mM 5-CQA or a 1.25 mM 3,5-diCQA solution, 50 μL of a 0.1% thiol aqueous suspension, and 150 μL of water.

A control reaction was carried out without CQA derivatives, beverages, or acetone powder. The thiol-capturing activity of each material was measured in duplicate. Capturing activity (percent) was expressed as $(C - P)/C \times 100$, where C is the amount of thiol compound in the control reaction and P is the amount of thiol compound in the reaction with CQA derivatives, beverages, or acetone powder.

(ii) Capture of AMS in Vivo. The effectiveness of removing bad breath by drinking a beverage was examined in a healthy 48-year-old male with no problem with halitosis. Beverages were prepared by extracting 2 g of ku-ding-cha powder or 4 g of green tea powder with 150 mL of hot water for 5 min. At lunch, the subject ate 480 g of cooked rice and a soup containing 9 g of garlic paste in 10 min, rinsed his mouth with a cup of water, and successively drank 150 mL of a beverage or ate 150 g of an apple (cv. Ourin) in 5 min. He breathed deeply and stopped his breath for 20 s, and then 3 L of his breath was collected in a polyester film bag, which is odorless and suitable for the preservation of gases, with each sampling over an 8 h period. Furthermore, he did not eat anything except 100 mL of water after each sampling time for 1−8 h. The concentrations of AMS gas in his breath were measured with a gas chromatograph. His breath was introduced into a 5 mL loop of a gas sampler (model MGS-5, Shimadzu Corp., Kyoto, Japan) and analyzed by a GC-14B (Shimadzu Corp.) equipped with a flame photometric detector (140 °C) and a glass column (polyphenyl ether 5 ring 10%, 3.2 mm × 3.1 m) according to the methods of Tamaki and Sonoki (*21*). Column temperature was held at 50 °C for 3 min, raised from 50 to 150 °C at 20 °C/min, and then held at 150 °C for 14 min. Helium gas (60 mL/min) was used as a carrier gas. Retention times of MeSH, MeSMe, AllSH, AMS, $(MeS)_2$, AllSAll, and $(AllS)_2$ standard samples were 1.6, 2.4, 4.1, 5.8, 7.0, 8.3, and 13.3 min, respectively. The beverages and apple were assayed on different days and in duplicate.

Large-Scale Reaction between 3,5-DiCQA and MeSH and Separation of Conjugates. The reactions were done on a large scale to identify the reaction products between 3,5-diCQA and MeSH. The reaction mixture contained 200 mg of apple AP, 20 mL of 0.1 M acetate buffer (pH 5.0), 20 μL of 15% MeSNa, and 20 mg of crude 3,5-diCQA. The mixture was stirred for 40 min at 25 °C. Twenty microliters of a 15% MeSNa solution was added at 1, 3, 5, 10, 20, and 30 min after the reaction started. The reaction was stopped by adding 2.0 mL of a 2 N HCl. The reaction was repeated five times. The reaction products were purified by using a Wakosil 25C18 column according to the methods described for the isolation of 3,5-diCQA. A stepwise elution with MeOH from 30 to 60% was carried out. The eluates were analyzed by HPLC, and the main products were eluted with 50% MeOH and

1% AcOH. The fraction containing the conjugates was concentrated and lyophilized to yield 12.3 mg of powder. Five milligrams of this powder was analyzed by NMR.

Quantitative Analysis of CQA Derivatives and Catechins in Beverages and Plants. CQA derivatives or catechins were extracted twice from 1.0 g of powder of a product for beverage with 90 mL of hot water for 5 min. The mixture was filtered through a filter paper under suction, and the filtrate was made up to 200 mL with water. On the other hand, CQA derivatives from 1.0 g of powder of a lyophilized or dried plant material were extracted three times with 100 mL of 80% acetone. This mixture was well dispersed by ultrasonic wave treatment for 5 min, followed by standing for 50 min. Each mixture was filtered through a filter paper under suction and the combined filtrate concentrated to <2 mL in a rotary vacuum evaporator at 45 °C. Water was added to this solution, and chlorophyll was removed by extraction with *n*-pentane. After removal of *n*-pentane by further concentration, the extract was made up to 100 mL with 50% MeOH. Each 5 μL of the solutions prepared from beverages and plant materials was analyzed by HPLC. Although no authentic samples of 3-CQA, 4-CQA, 3,4-diCQA, and 4,5-diCQA were available, we identified these compounds by LC-MS analysis and comparison to HPLC patterns of other researchers (*29*). The amounts of mono- and di-CQA were calculated by using 5-CQA and 3,5-diCQA, respectively, as standards.

HPLC Analysis. Phenolic compounds and conjugates with MeSH were analyzed by HPLC (LC-10AD system controlled by CLASS-VP software ver. 6.1, Shimadzu Corp., Kyoto, Japan) with a TSKgel ODS-80Ts column (4.6 × 250 mm, 5 μm, Tosoh Corp., Tokyo, Japan) at 30 °C. Elution was performed with MeOH/1% AcOH (a linear gradient from 20 to 44% MeOH for 50 min and holding at 44% MeOH for 20 min) at a flow rate of 0.6 mL/min. Eluates were monitored at 280 and 330 nm and their spectra measured using a photodiode array (PDA), SPD-M10Avp (Shimadzu Corp.). Six CQA derivatives [3-CQA (10.1 min), 5-CQA (16.3 min), 4-CQA (17.3 min), 3,4-diCQA (42.4 min), 3,5-diCQA (43.8 min), 4,5-diCQA (53.1 min)], 4 catechins [(−)-epigallocatechin (EGC, 11.5 min), (−)-epigallocatechin gallate (EGCG, 16.8 min), (−)-epicatechin (EC, 21.0 min), (−)-epicatechin gallate (ECG, 29.7 min)], caffeic acid (21.9 min), and caffeine (19.3 min) in beverages and plants were separated and determined. The analysis was carried out in duplicate, and the values were averaged.

LC-MS Analysis. LC-MS analysis was carried out using a Waters LC-MS system (Waters Corp., Milford, MA) equipped with a Waters ZQ mass detector, a Waters 2690 separations module, a Waters 996 PDA detector, and MassLynx software version 3.5. Column and chromatographic conditions were the same as those used for the above HPLC analysis. The postcolumn split was 4:1. ESI mass spectrometry was used for the detection of CQA derivatives and the conjugates. The following were the MS parameters: ionization mode, ES^+; scan range, *m*/*z* 50−800; scan rate, 0.5 s; capillary voltage, 3.4 kV; cone voltage, 15 V; source block temperature, 120 °C; and desolvation temperature, 400 °C. Nitrogen was used as desolvation and cone gas at flows of 400 and 50 L/h, respectively.

NMR Spectrum. The structures of the isolated compounds were elucidated by measuring their NMR spectra in CD_3OD with a JNM-A400 spectrometer (JEOL, Tokyo, Japan). The methyl resonance of TMS at δ 0 in CD_3OD was used as an internal standard.

RESULTS AND DISCUSSION

CQA Derivatives in Beverages and Plants. Six CQA derivatives, 3-CQA, 4-CQA, 5-CQA, 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA, in ku-ding-cha were each separated by HPLC as shown in **Figure 1**. 3,5-DiCQA was isolated from ku-ding-cha by extraction and C18 column chromatography and identified by NMR. 3-CQA, 4-CQA, 5-CQA, 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA were also identified by LC-MS and the elution order in HPLC compared to other studies (*24*−*29*). The spectra of other minor peaks also had an absorption maximum near 330 nm, characteristic of caffeic acid moiety. **Table 1** shows the contents of the CQA derivatives from the products of several beverages and related raw plant materials. Compared to coffee beans (roasted), ku-ding-cha, except for ku-ding-cha 4, contained very large amounts of CQA derivatives. Ku-ding-cha 3 contained 6.3 g of 5-CQA, 10.6 g of 3,5-diCQA, and 4.3 g of 4,5-diCQA in 100 g of dry weight. Smaller quantities of other CQAs were present. The total CQA derivatives corresponded to 90.3 mmol as caffeic acid moiety. It was reported that several species, especially *Ilex* species, have been used to produce ku-ding-cha in China (*1*, *9*, *10*). Therefore, we analyzed several plants of *Ilex* species and mate in addition to ku-ding-cha. The results indicate that *I. latifolia*, *I. rotunda*, and mate (*I. paraguariensis*) contain a large amount of total CQA derivatives, 63.9, 73.8, and 42.1 mmol as caffeic acid moiety, respectively, as compared to green coffee beans at 22.3 mmol. On the other hand, a small amount of caffeic acid was detected in ku-ding-cha 3 (0.10 mmol/100 g of dry weight), mate (0.12 mmol/100 g), *I. latifolia* (0.45 mmol/100 g), *I. integra* (0.07 mmol/100 g), *I. rotunda* (0.10 mmol/100 g), *I. cornuta* (0.05 mmol/100 g), and others (<0.04 mmol/100 g). In the materials shown in **Table 1**, no catechins could be detected. Furthermore, caffeine was detected in mate (1.43 g/100 g) as well as coffee (roasted, 1.35 g/100 g; green, 1.36 g/100 g from 80% acetone extract).

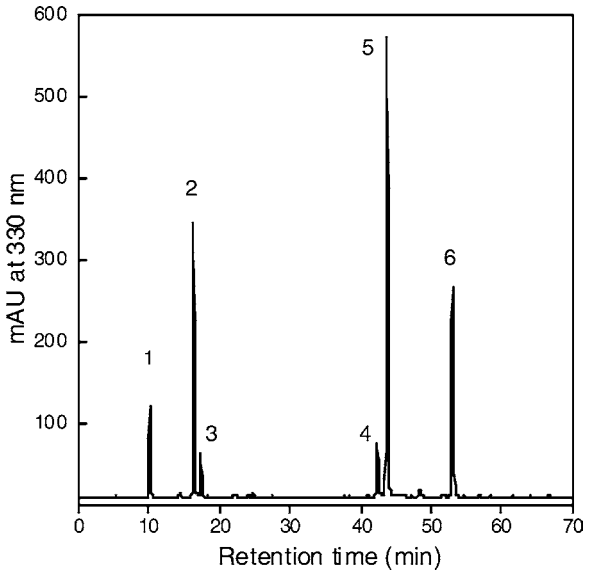


Figure 1. Separation of several CQA derivatives in ku-ding-cha by HPLC: 1, 3-CQA; 2, 5-CQA; 3, 4-CQA; 4, 3,4-diCQA; 5, 3,5-diCQA; 6, 4,5-diCQA.

Thiol-Capturing Activities of CQA Derivatives and Beverages. The analysis of ku-ding-cha and our studies on deodorization (*11*−*16*) have suggested that a very large amount of 5-CQA and 3,5-diCQA in ku-ding-cha, mate, and coffee can contribute to removal of bad odor from the environment. The capturing activities of these compounds toward thiols are shown in **Table 2**. No activity of 5-CQA or 3,5-diCQA alone appeared; however, by their combination with apple AP, high capturing activities were revealed toward MeSH and PrSH. If the reaction time and amount of apple AP is large, AllSH-capturing activity is thought to become high (*12*). In **Table 2**, the amount of caffeic acid moiety was the same in both tests with 5-CQA and 3,5-diCQA, because the reaction site involved in the oxidation reaction of polyphenol oxidase (PPO) and captured thiols is the caffeic acid moiety. Therefore, this demonstrated that the thiol-capturing activity of 5-CQA with apple AP is stronger than that of 3,5-diCQA with apple AP. **Tables 3** and **4** show the content of catechins in green, black, and oolong teas, and the thiol-capturing activities of several beverages, respectively. Ku-ding-cha 1 also needs AP containing PPO to present sufficient activity

Table 1. Content of Caffeoyl Quinic Acid Derivatives in Several Products for Beverages and Raw Plant Materials

material	contents (g/100 g of dry wt)						total CA[d] (mmol)
	3-CQA	4-CQA	5-CQA	3,4-diCQA	3,5-diCQA	4,5-diCQA	
beverage product[a]							
ku-ding-cha 1	0.15	0.18	1.45	0.12	3.75	0.62	22.4
ku-ding-cha 2	0.96	0.64	1.82	0.99	5.44	2.97	46.0
ku-ding-cha 3	1.70	1.13	6.32	1.81	10.60	4.25	90.3
ku-ding-cha 4	0.61	0.47	1.43	0.05	0.54	0.18	10.1
mate (*I. paraguariensis*)	2.68	1.22	2.30	0.60	4.04	1.72	42.1
coffee bean, roasted (*C. arabica*)	0.60	0.68	1.35	0.07	0.05	0.08	8.2
raw plant material[b]							
I. latifolia[c]	4.27	0.12	4.80	0.07	9.46	0.28	63.9
I. integra[c]	2.79	0.14	4.41	0.01	0.71	0.07	23.8
I. rotunda[c]	1.88	0.04	3.76	0.09	14.57	0.28	73.8
I. macropoda[c]	0.11	0.01	2.50	0.06	4.98	0.33	28.2
I. cornuta[c]	0.59	0.03	2.23	0.06	4.96	0.12	27.9
coffee bean, green (*C. arabica*)	0.40	0.61	5.54	0.16	0.39	0.43	22.3

[a] Products were dried matter. Ku-ding-chas 1–4 were different from each other in their shapes. [b] All were lyophilized except for coffee beans, which were dried. [c] The leaves of the plants were used. [d] Total caffeic acid (CA) moiety (mmol/100 g of dry wt) in the above six CQA derivatives.

Table 2. Thiol-Capturing Activities of CQA Derivatives

substance[a]	capturing activity (%)		
	MeSH	PrSH	AllSH
5-CQA	4	9	2
5-CQA + apple AP[b]	100	100	45
3,5-diCQA	0	14	0
3,5-diCQA + apple AP[b]	72	83	19

[a] Two micromoles of 5-CQA and 1 μmol of 3,5-diCQA were used to measure thiol-capturing activity. [b] Two milligrams (PPO, 6.2 units) of apple acetone powder (AP) was used.

Table 3. Content of Catechins in Green, Black, and Oolong Teas

tea	contents (g/100 g of dry wt)				total c and p[a] (mmol)
	EGC	EGCG	EC	ECG	
green	4.32	5.95	1.01	1.23	49.2
black	2.01	1.45	0.13	1.20	18.9
oolong	1.80	3.60	0.54	0.85	27.4

[a] Total catechol and pyrogarol moiety (mmol/100 g of dry wt) in the above four catechins.

Table 4. Thiol-Capturing Activities of Several Beverages

beverage[a]	capturing activity (%)		
	MeSH	PrSH	AllSH
ku-ding-cha 1	3	21	7
ku-ding-cha 1 + apple AP	100	100	100
green tea	0	13	7
green tea + pear AP	27	26	23
black tea	0	4	0
black tea + pear AP	34	30	30
oolong tea	0	19	4
oolong tea + pear AP	31	30	44

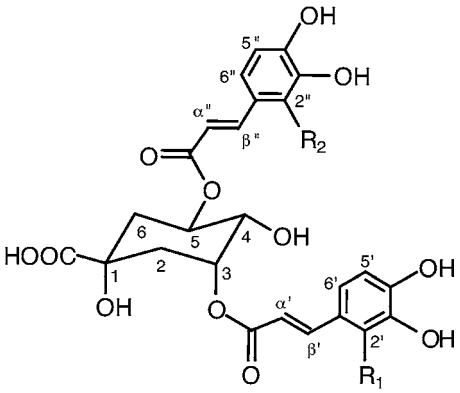
[a] Ku-ding-cha 1, green tea, black tea, and oolong tea contained 9.0, 19.7, 7.6 and 11.0 μmol, respectively, of catechol and pyrogarol moiety, calculated from **Tables 1** and **3**.

to capture thiols. The removal of MeSH by tea catechins without AP has been reported by other researchers (*30*). However, the activities of green tea toward thiols were not strong in our experiments, even in the presence of a large amount of AP (or PPO activity). Apple AP (10 mg) with 31 units of PPO activity for 5-CQA and pear AP (20 mg) with 175 units of PPO activity for EC were used for ku-ding-cha 1 and three teas, respectively. On the other hand, ku-ding-cha 1 indicated extremely strong activities toward three kinds of thiols in the presence of apple AP. This suggests that ku-ding-cha containing a large amount of CQA derivatives exerts an effect on the removal of bad odors with a very small amount of catalysts such as PPO and weak alkaline materials such as sodium hydrogen carbonate (*14*). In addition, the AllSH-capturing activity of oolong tea with pear AP was stronger than that of other beverages belonging to *Camellia sinensis*, which contains large amounts of catechins (*31*; **Table 3**). This is thought to link to a low ascorbic acid content (*32*). Ascorbic acid appears to suppress the deodorizing activity of PPs against MeSH (*33*). The deodorizing activity of green tea may have been weak, because green tea contains much ascorbic acid.

Identification of Conjugates between 3,5-DiCQA and MeSH. Previously we isolated 2-methylthiochlorogenic acid, which is a conjugate between 5-CQA and MeSH, and demonstrated a mechanism for thiol capture (*11*). As it was demonstrated in the amount of caffeic acid moiety that 3,5-diCQA has a similar level of thiol-capturing activity as 5-CQA, we tried to identify the reaction products between 3,5-diCQA and MeSH. We obtained two conjugates as a mixture, which were separated at the retention times of 54.1 and 55.9 min and at a ratio of about 1:1 by HPLC with the conditions described under Materials and Methods. This fraction did not contain any 3,5-diCQA. In ESI mass spectra of both peaks, $[M + H]^+$ at *m*/*z* 563, $[M + Na]^+$ at *m*/*z* 585, and $[(M + H) - H_2O]^+$ at *m*/*z* 545 were detected as compared to $[M + H]^+$ at *m*/*z* 517, $[M + Na]^+$ at *m*/*z* 539, and $[(M + H) - H_2O]^+$ at *m*/*z* 499 in the ESI mass spectrum of 3,5-diCQA. The NMR spectra were compared to those of 2-methylthiochlorogenic acid (*11*) and 3,5-diCQA. In the ^{1}H NMR spectrum, four sets of signals were derived from caffeic acid moieties (**Table 5**). In the two caffeic acid moieties, the 2-positions of aromatic rings were substituted by SMe (**Table 5**, CA-3 and CA-4). In the ^{13}C NMR spectrum, two methyl signals at δ 19.25 and 19.33 were also observed. Furthermore, three signals of H-2 and H-6, H-4, and H-3 and H-5 in quinic acid moieties were observed at δ 2.17−2.34 (8H, m), 3.97 (2H, dd), and 5.43 (4H, m), respectively. These data confirm that the conjugates consist of two kinds of 3,5-diCQA adding one SMe at the 2-position of either aromatic ring. As it is known that the H-α signal shifts to a lower field with the substitution of caffeic acid to 3-OH of the quinic acid moiety rather than to 5-OH (*27*, *34*), structures combining quinic acid

Table 5. ^{1}H NMR Signals of Caffeic Acid Moieties in a Mixture of Conjugates between 3,5-DiCQA and MeSH

caffeic acid moiety	chemical shift (δ) [coupling constant (J, Hz)]					
	C=C		aromatic ring			
	H-α' (α'')	H-β' (β'')	H-2′ (2″)	H-5′ (5″)	H-6′ (6″)	–S-Me
CA-1	6.36 (d, 15.9)	7.62 (d, 15.9)	7.07 (d, 2.1)	6.78 (d, 8.2)	6.97 (dd, 1.5, 8.1)	
CA-2	6.27 (d, 15.9)	7.58 (d, 15.9)	7.06 (d, 1.8)	6.78 (d, 8.2)	6.97 (dd, 1.8, 8.1)	
CA-3	6.45 (d, 16.2)	8.47 (d, 16.2)		6.84 (d, 8.5)	7.25 (d, 8.5)	2.292 (s)
CA-4	6.37 (d, 15.9)	8.44 (d, 15.9)		6.84 (d, 8.5)	7.24 (d, 8.5)	2.287 (s)



	R_1	R_2
Adduct 1 :	H	SCH_3
2 :	SCH_3	H

Figure 2. Structures of conjugates of 3,5-diCQA and MeSH.

with CA-1 and CA-4 and with CA-2 and CA-3 (**Table 5**) are assigned to adducts 1 and 2 (**Figure 2**), respectively.

In Vivo Deodorization. Ku-ding-cha is recognized to have a high thiol-capturing activity catalyzed by PPO in vitro. Furthermore, we investigated the effect of ku-ding-cha on the removal of AMS, which is formed in the gut and causes the persistence of malodorous breath (*18*) when garlic is consumed.

Ku-ding-cha 1 (**Table 1**), green tea (**Table 3**), or apple (cv. Ourin) was separately fed to a subject after the consumption of garlic. In this experiment, thiols decreased quickly to levels that were not detected by GC; however, AMS was detected at significant levels as was also reported by Rosen et al. (*19*). **Figure 3** shows the time course of AMS decrease. The presence of AMS early after garlic ingestion is thought to form in the oral cavity, the throat, and the esophagus and later to be released from the lungs after being absorbed in the intestine (*18*). When an apple was eaten by a subject as a deodorant material, the amount of AMS decreased significantly 1 h after garlic ingestion as compared to the control in which a subject drank water instead of the deodorant material. However, the amount of AMS increased 2−4 h after garlic ingestion and became about the same or more as compared to the control experiment. This indicates that PPO in apple is not important in vivo. In this case, deodorization with apple is attributed to its polyphenol content. In ku-ding-cha, ∼10 ppb of AMS was less than the control during 3−5 h after garlic ingestion, whereas in green tea the amount of AMS was about the same as in the control experiment. In this experiment, we used 2 g of ku-ding-cha 1 and 4 g of green tea, which contain 0.45 mmol of caffeic acid moiety and 1.97 mmol of catechol and pyrogarol moieties,

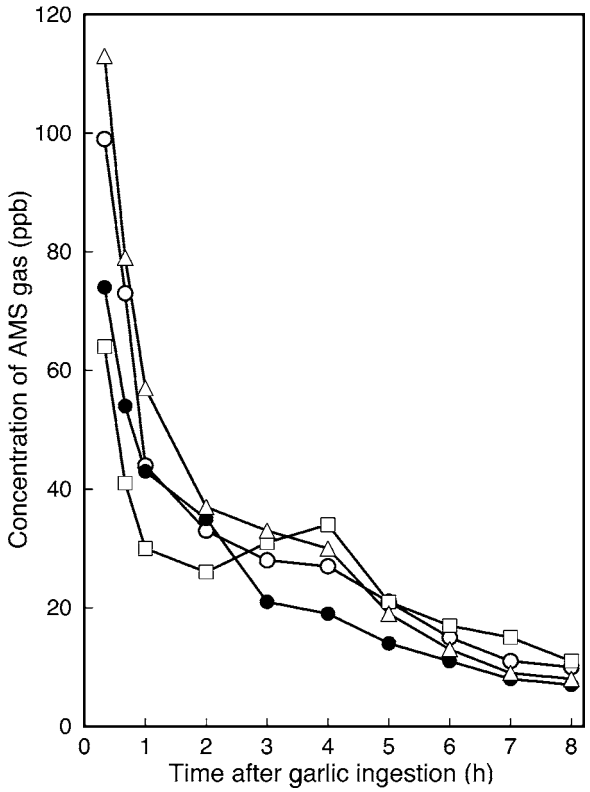


Figure 3. Effects of ku-ding-cha 1, green tea, and apple on capture of AMS in vivo: (○) control (water); (●) ku-ding-cha 1; (△) green tea; (□) apple (cv. Ourin).

respectively. These suggest that ku-ding-cha 1 is superior to green tea in deodorization. Although the decreased amount of AMS was small, these results indicate a good effect of ku-ding-cha on deodorization. The beverage was fed to a subject only once after the ingestion of garlic in this investigation. If the beverage were consumed frequently, the effect on deodorization might be more remarkable. The early decrease in AMS by eating an apple is likely to be a result of decrease in AllSH by enzymatic deodorization (*15*), because AMS has been reported to be formed by the methylation of AllSH (*22*). However, this effect does not continue because an apple does not contain as much PPs as ku-ding-cha (*35*). Although PPs have a weak ability to capture sulfides and disulfides (*16*), PPs in ku-ding-cha probably capture large amounts of thiols produced from sulfides and disulfides in the intestine. As a result, formation of AMS gas is suppressed. Bad odors of the mouth and body may be decreased by drinking ku-ding-cha, mate, or coffee, which contain large amounts of PPs such as chlorogenic acid derivatives, as well as teas, which contain large amounts of catechins (*31*) and less ascorbic acid (*32*). These may be classified as catechin teas and chlorogenic acid teas. Furthermore, our results suggest that the combination of eating an apple and drinking a chlorogenic acid tea such as ku-ding-cha may be most effective for deodorization after garlic ingestion.

ABBREVIATIONS USED

AMS, allyl methyl sulfide; AP, acetone powder; CA, caffeic acid; CQA, caffeoyl quinic acid; diCQA, dicaffeoyl quinic acid; EC, (−)-epicatechin; ECG, (−)-epicatechin gallate; EGC, (−)-epigallocatechin; EGCG, (−)-epigallocatechin gallate; ESI, electrospray ionization; PDA, photodiode array; POD, peroxidase; ODS, octadecyl silicone; PP, polyphenolic compound; PPO, polyphenol oxidase; TMS, tetramethylsilane.

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Received for review February 24, 2004. Revised manuscript received June 8, 2004. Accepted June 13, 2004.

JF049693J