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# Enzymatic Preparation of Kaempferol from Green Tea Seed and Its Antioxidant Activity

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Among the flavonols in green tea, kaempferol has many biological activities but kaempferol of plant origin is too expensive to be used in commercial products. Recently, we confirmed that green tea seed (GTS) contained a reasonable amount of kaempferol glycoside. After conducting structure analysis, two kaempferol glycosides were identified, kaempferol-3-*O*-[2-*O*-$\beta$-D-galactopyranosyl-6-*O*-$\alpha$-L-rhamnopyranosyl]-$\beta$-D-glucopyranoside (compound **1**) and kaempferol-3-*O*-[2-*O*-$\beta$-D-xylopyranosyl-6-*O*-$\alpha$-L-rhamnopyranosyl]-$\beta$-D-glucopyranoside (compound **2**), respectively. Also, a commercially useful method for kaempferol preparation was suggested by enzymatic hydrolysis using these two flavonoids. After several enzyme reactions were performed for the complete bioconversion of compounds **1** and **2** to kaempferol, we found that the optimum enzyme combination was reaction with $\beta$-galactosidase and hesperidinase. Finally, we produced pure kaempferol with over 95% purity. We also compared the antioxidant effect of these two GTS flavonoids and its aglycone, kaempferol. Kaempferol is a more efficient scavenger of 1,1-diphenyl-2-picrylhydrazyl radicals and a better inhibitor of xanthine/xanthine oxidase than the two glycosides.

**KEYWORDS: Tea seed; enzymatic hydrolysis; antioxidant; kaempferol; kaempferol glycoside**

## INTRODUCTION

In recent years, cosmetic, pharmaceutical, and chemical industries have become increasingly interested in antioxidants (*1*). Recent research efforts on antioxidants have focused on flavonoids that show strong free radical scavenging effects and metal ion chelating properties. In addition to their antioxidant activity, flavonoids have been reported to inhibit various enzymes such as cyclooxygenase and lipoxygenase related to inflammation (*2*). Evidence for the presence of flavonoids in ancient remedies for burns and inflammation has been reported, and these substances, which have been isolated, are presently used in commercial products (*3*, *4*). Thus, dietary flavonoids have attracted attention for potential beneficial effects on humans.

Tea [*Camellia sinensis* (L.) O. Kuntze, Theaceae] has been cultivated widely in Asia for centuries. Epidemiological studies suggest that the consumption of tea provides protection against cancers in humans. Tea is generally consumed in one of three forms: green, oolong, or black. Approximately 3.0 million metric tons of dried tea is produced annually, of which 20% is green tea, 2% is oolong, and the remainder is black tea (*5*).

To produce green tea, after the leaves are picked, the young leaves are rolled and steamed to minimize oxidation (*6*). Recently, as the popularity of green tea has increased, the production of green tea seed (GTS) has also increased. As tea contains a number of chemical constituents possessing medicinal and pharmacological properties, GTS contains many biologically active compounds such as saponins, flavonoids, vitamins, and oils (*7*).

The major polyphenols present in teas are the flavan-3-ols and flavonols. Flavan-3-ols form about 77% of phenolic compounds in green tea, while flavonols make up 13%. Therefore, flavan-3-ols have attracted more attention from researchers. However, flavonols are structurally more stable than flavan-3-ols, and it has been shown that tea is a major dietary source of these compounds. The main flavonols in tea are glycosides of quercetin and kaempferol with lower levels of myricetin (*7*). Concerning the commercial use of flavonols for other industrial purposes, the production of pure separated flavonols from plant sources such as tea is not easy.

In this study, we report the preparation of kaempferol by enzymatic hydrolysis of green tea seed extract (GTSE). For the optimum enzymatic hydrolysis of GTSE, several glycosylation enzymes were investigated. Also, the antioxidative effect of kaempferol was compared with its glycosides.

## MATERIALS AND METHODS

**Materials.** GTS [*C. sinensis* (L.) O. Kuntze, Theaceae] was purchased from Zhejiang Chemicals, China. The following enzymes and chemicals were obtained from Sigma Chemical Co. (St. Louis, MO): a hesperidinase from *Penicillium* sp. (18 units/g), a $\beta$-glucosidase from almonds (2.4 units/mg), a cellulase from *Aspergillus niger* (5.1 units/mg), a $\beta$-glucuronidase from *Helix pomatia* (338 units/mg), a

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pectinase from *Rhizopus* sp. (490 units/g), a $\beta$-galactosidase from *Aspergillus oryzae* (8.7 units/mg), an amyloglucosidase from *A. niger* (51 units/mg), an $\alpha$-amylase from human saliva (type XIII, A, 40 units/mg), a dextranase from *Penicillium* sp. (425 units/mg), a $\beta$-xylosidse from *A. niger* (8.5 units/mg), a xylanase from *Thermomyces lanuginosus* (285 units/mg), kaempferol, 1,1-diphenyl-2 -picrylhydrazyl (DPPH), sodium nitroprusside, xanthine, xanthine oxidase (XO) grade I from buttermilk (EC 1.1.3.22), phenazine methosulfate, $\beta$-nicotinamide adenine dinucleotide, and nitroblue tetrazolium chloride (NBT). All organic solvents used were of analytical grade and purchased from Fisher Scientific UK (Loughborough, Leics, United Kingdom).

**High-Performance Liquid Chromatography (HPLC) and Thin-Layer Chromatography (TLC) Analysis.** The analysis of extracts and enzyme reactants was carried out by the following HPLC and TLC methods. The HPLC system consisted of a Waters 2695 separation module and a 2996 photodiode array (PDA) detector. A 250 mm × 4.6 mm i.d. Mightysil C18 reverse phase column (Kanto Chemical, Japan) was employed. The detector wavelength was set at 263 nm. The mobile phase used for the analysis of samples was a mixture of distilled water (A) and acetonitrile (B) with gradient elution. The gradient elution was 15−80% B in 60 min at a flow rate of 1 mL/min. TLC analysis was performed on Merck Kieselgel 60 $F_{254}$ silica gel plates using *n*-butanol−acetic acid−water (3:1:1, v/v/v). The separated hydrolysates on TLC plates were visualized by dipping the plates into 5% (v/v) $H_2SO_4$ in methanol containing 0.3% (w/v) *N*-(1-naphthyl) ethylenediamine, followed by drying and heating for 10 min at 121 °C.

**Liquid Chromatography/Mass Spectrometry (LC/MS) and NMR Analysis.** LC/MS-atmospheric pressure chemical ionization (APCI) analyses were made using a Thermo Finnigan (Surveyor) HPLC with a PDA detector coupled to a Thermo Finnigan (LCQ Classic) quadrupole ion trap mass spectrometer equipped with an APCI source. Chromatographic separations of material were performed on a 250 mm × 4 mm i.d., 5 $\mu$m, Merck LiChrosphere C18 column using a 1 mL/min solvent gradient of 20−100% aqueous methanol (containing 1% acetic acid) in 40 min. The MS interface was set to positive ion mode [vaporizer tube temperature, 550 °C; needle current, 5 $\mu$A (approximately 3.6 kV); sheath and auxiliary nitrogen gas pressures, 80 and 20 psi; and heated capillary temperature, 150 °C]. Mass spectra were acquired in the range of (full) *m/z* 125−1200 (MS-MS ion isolation width, 5 Da; MS-MS collision energy, 45%; and MS-MS scan range, *m/z* of parent ion to ca. 1/3 *m/z* parent ion). To determine the structure of the isolated compound from GTSE, $^{1}H$, $^{13}C$ NMR spectra were recorded on a Varian GEMINI-300BB (300 MHz) spectrometer.

**Isolation of Kaempferol Glycosides from GTS.** GTS was ground for extraction in a FM-680T grinder (Food Mixer, Han il, Korea), and crushed GTS (100 g) was defatted three times with *n*-hexane (3 L) for 3 h. After the solvent was removed by filtration, defatted and dried GTS (78.5 g) was extracted with 2 L of 70% ethanol in a Soxhlet apparatus for approximately 6 h and was then filtered. Evaporation of the solvent under reduced pressure provided the GTS extract (11.2 g). The GTS extract (11.2 g) was subjected to a medium-pressure liquid chromatography (MPLC) system (Yamazen Co. Japan) using a gradient elution system of distilled water and acetonitrile gradient with acetonitrile from 15 to 50% acetonitrile in 60 min at a flow rate of 30 mL/min. The pressure of the system was 3 Mpa. A 300 mm × 37 mm i.d, 50 $\mu$m, Ultra pack-ODS-S-50C column (Yamazen Co., Japan) was used. The detector wavelength was set at 263 nm. The fraction volume was 30 mL. Ten fractions were collected and monitored by HPLC. The fractions were combined [fr. 1−4 (7.5 g), fr. 5 (0.25 g), fr. 6 and 7 (1.9 g), fr. 8 (0.31 g), and fr. 9 and 10 (1.24 g)], and compounds **1** (fr. 5) and **2** (fr. 8) were isolated in yields of 0.25 and 0.31 g, respectively. To determine the structure of the isolated compounds, LC/MS and NMR analyses were conducted. Acid hydrolysis was conducted for the analysis of carbohydrate residues.

**Compound 1.** LC/MS *m/z* 756.9 [M + H]. NMR $\delta_H$ ($CD_3OD$, 300 MHz): 8.02 (2H, d, $J$ = 8.7 Hz, H-2′, H-6′), 6.90 (2H, d, $J$ = 9.0 Hz, H-3′, H-5′), 6.37 (1H, d, $J$ = 1.8 Hz, H-8), 6.17 (1H, d, $J$ = 1.8 Hz, H-6), 5.33 (1H, d, $J$ = 7.8 Hz, H1-Glc), 4.70 (1H, d, $J$ = 7.5 Hz H1-Gal), 4.47 (1H, d, $J$ = 0.9 Hz H1-Rha), 3.2−3.8 (16H, m), 1.08 (3H, d, $J$ = 6.3 Hz). NMR $\delta_C$ ($CD_3OD$, 75 MHz): 179.48 (C-4), 166.59 (C-9), 163.02 (C-7), 161.47 (C-5), 159.25 (C-2), 158.60 (C-4′), 134.72 (C-3), 132.37 (C-2′, 6′), 122.94 (C-1′), 116.24 (C-3′, 5′), 105.57 (C-10), 104.48 ($C_{Gal}$-1), 101.12 ($C_{Glu}$-1), 102.21 ($C_{Rha}$-1), 100.19 (C-6), 95.06 (C-8), 82.05 ($C_{Glu}$-2), 78.28 ($C_{Glu}$-3), 77.87 ($C_{Gal}$-5), 77.82 ($C_{Glu}$-5), 76.95 ($C_{Gal}$-3), 75.38 ($C_{Gal}$-2), 73.86 ($C_{Rha}$-3), 72.30 ($C_{Rha}$-2), 72.07 ($C_{Glu}$-4), 71.40 ($C_{Rha}$-4), 71.27 ($C_{Gal}$-4), 69.71 ($C_{Rha}$-5), 68.22 ($C_{Glu}$-6), 62.59 ($C_{Gal}$-6), 17.85 ($C_{Rha}$-6). All of these data corresponded to those in the literature for camelliaside A (*7*).

**Compound 2.** LC/MS *m/z* 726.9 [M + H]. NMR $\delta_H$ ($CD_3OD$, 300 MHz): 8.00 (2H, d, $J$ = 8.7 Hz, H-2′, H-6′), 6.80 (2H, d, $J$ = 9.0 Hz, H-3′, H-5′), 6.34 (1H, d, $J$ = 2.1 Hz, H-8), 6.14 (1H, d, $J$ = 2.1 Hz, H-6), 5.35 (1H, d, $J$ = 7.2 Hz, H1-Glc), 4.73 (1H, d, $J$ = 7.5 Hz H1-Xyl), 4.45 (1H, d, $J$ = 1.2 Hz H1-Rha), 3.2−3.8 (15H, m), 1.09 (3H, d, $J$ = 6.3 Hz). NMR $\delta_C$ ($CD_3OD$, 75 MHz): 179.43 (C-4), 165.91 (C-9), 163.04 (C-7), 161.36 (C-5), 158.70 (C-2), 158.47 (C-4′), 134.83 (C-3), 132.35 (C-2′, 6′), 122.92 (C-1′), 116.17 (C-3′, 5′), 105.73 (C-10), 105.13 ($C_{Xyl}$-1), 100.83 ($C_{Glu}$-1), 102.14 ($C_{Rha}$-1), 99.92 (C-6), 94.85 (C-8), 81.93 ($C_{Glu}$-2), 78.21 ($C_{Glu}$-3), 77.05 ($C_{Xyl}$-3), 76.84 ($C_{Glu}$-5), 74.68 ($C_{Xyl}$-2), 73.85 ($C_{Rha}$-3), 72.27 ($C_{Rha}$-2), 71.42 ($C_{Glu}$-4), 72.06 ($C_{Rha}$-4), 70.99 ($C_{Xyl}$-4), 69.70 ($C_{Rha}$-5), 68.11 ($C_{Glu}$-6), 66.54 ($C_{Xyl}$-5), 17.85 ($C_{Rha}$-6). All of these data corresponded to those in the literature for camelliaside B (*7*).

**Acid Hydrolysis of Compounds 1 and 2.** Compounds **1** (10 mg) and **2** (10 mg) dissolved in 1 mL of 2 M HCl were heated at 100 °C for 1 h in a sealed tube. The reactant was centrifuged and separated into insoluble (precipitant) and soluble parts (supernatant). The insoluble part was washed several times with distilled water and dried in vacuo. The soluble part was neutralized with 1 mL of 2 M NaOH and then stored frozen for analysis.

**Enzymatic Hydrolysis of GTSE.** GTSE (0.5 g) in 8 mL of 0.02 M sodium−acetate buffer (pH 5.0) with 2 mL of several glycolytic enzyme solutions was incubated with stirring at 37 °C for a fixed time. The amount of each enzyme was adjusted to provide a final concentration in the mixture of 50 units/g of GTSE. Each sample and blank were used as a reaction control. All samples were prepared in duplicate. After incubation, each aliquot (200 $\mu$L) was extracted with ethanol (800 $\mu$L) and centrifuged at 12000*g* (4 °C) for 10 min. Supernatants were transferred to new tubes and dried completely in vacuo. It was stored frozen for analysis.

**DPPH Radical Scavenging Assay.** The DPPH radical-scavenging assay was as follows. The reaction mixture containing various concentrations of the test samples, final concentrations of 10, 25, 50, 100, 500, and 1000 $\mu$M, and 0.2 mM DPPH methanolic solution was incubated at room temperature for 30 min in the dark after which the absorbance was measured at 517 nm (Jasco V-550 spectrophotometer). The scavenging activity was expressed as a percent as compared to control DPPH solution (100%). The synthetic antioxidant Trolox and L-ascorbic acid were included in experiments as a positive control.

**Assay of Superoxide Anion Generated by Xanthine Oxidase.** Superoxide anions were generated by the xanthine/XO system, following the described procedure. The reaction mixture consisted of xanthine (0.5 mM), NBT (0.5 mM), and test samples at concentrations of 31, 63, 125, 250, and 500 $\mu$M, in a final volume of 100 $\mu$L. Xanthine and NBT were dissolved in 200 mM phosphate buffer with 0.25 mM EDTA, pH 7.5. The reaction mixture was preincubated at room temperature for 2 min and initiated by the addition of 100 $\mu$L of XO (50 mU/mL). The mixture (200 $\mu$L) was kept for 30 min at 37 °C. To detect superoxide, the coloring reagent [final concentration of 300 $\mu$g/mL sulfanilic acid, 5 $\mu$g/mL of *N*-(1-naphthyl)ethylenediamine dihydrochloride, and 16.7% (v/v) acetic acid] was added after the incubation. The mixture was allowed to stand for 30 min at room temperature, and the absorbance at 550 nm was measured (Ceres UV 900 Hdi). Different concentrations of compounds were analyzed, and then, the half-minimal inhibitory concentration ($IC_{50}$) was calculated by linear regression analysis.

**Assay of Uric Acid Generated by Xanthine Oxidase.** The effect of compounds on xanthine oxidase activity was evaluated by measuring the formation of uric acid from xanthine. The reaction mixtures contained the same proportion of components as in assay for superoxide anion, except NBT. The reaction mixture consisted of xanthine (0.5 mM), dissolved in 200 mM phosphate buffer with 0.25 mM EDTA,

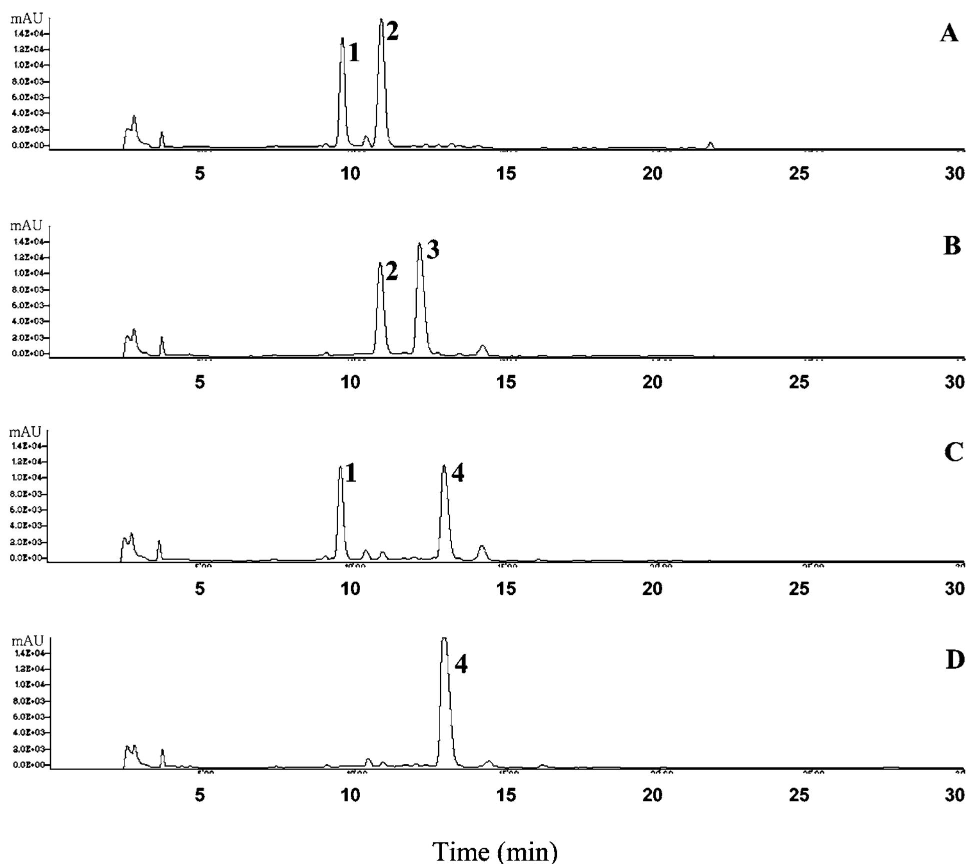



**Figure 1.** HPLC profile of GTSE and the hydrolysis of GTSE with the commercial glycolytic enzymes. (**A**) GTSE, (**B**) reaction with hesperidinase, (**C**) reaction with cellulase, and (**D**) reaction with $\beta$-galactosidase. Key to peak identity: 1, kaempferol-3-*O*-[2-*O*-$\beta$-D-galactopyranosyl-6-*O*-$\alpha$-L-rhanmopyranosyl]-$\beta$-D-glucopyranoside; 2, kaempferol-3-*O*-[2-*O*-$\beta$-D-xylopyranosyl-6-*O*-$\alpha$-L-rhanmopyranosyl]-$\beta$-D-glucopyranoside; 3, kaempferol-3-O-$\beta$-D-galactopyranosyl (1→2)-$\beta$-D-glucopyranoside; and 4, kaempferol-3-O-$\alpha$-L-rhanmopyranosyl (1→6)-$\beta$-D-glucopyranoside.

pH 7.5, and test samples at concentrations of 31, 63, 125, 250, and 500 $\mu$M, in a final volume of 100 $\mu$L. The reaction mixture were preincubated at room temperature for 2 min and initiated by the addition of 100 $\mu$L of XO (50 mU/mL). The mixture (200 $\mu$L) was kept for 30 min at 37 °C. The reaction was stopped with HCl (20 $\mu$L, 5 M). The production of uric acid was determined specrophotometically at 295 nm (Ceres UV 900 Hdi).

## RESULTS AND DISCUSSION

**Purification and Identification of Compounds in GTS.** As shown in **Figure 1A**, HPLC analysis of GTSE gives just two peaks, compounds **1** and **2**. For the structure analysis of these compounds, GTSE was further purified by MPLC. The fractions were combined after monitoring HPLC, and compounds **1** and **2** were isolated in yields of 250 (0.25%) and 310 mg (0.31%), respectively. To determine the structure of the isolated compounds **1** and **2**, acid hydrolysis, LC/MS, and NMR analysis were conducted. Compound **1** was obtained as a pale yellow amorphous powder. Upon acid hydrolysis of **1**, kaempferol, galactose, glucose, and rhamnose were identified by TLC. The $^{1}$H NMR spectrum showed four kinds of aromatic protons [$\delta$ 8.02 (2H, d, $J$ = 8.7 Hz, H-2′, H-6′), 6.90 (2H, d, $J$ = 9.0 Hz, H-3′, H-5′), 6.37 (1H, d, $J$ = 1.8 Hz, H-8), and 6.17 (1H, d, $J$ = 1.8 Hz, H-6)] indicating the kaempferol skeleton with three anomeric protons [$\delta$ 5.33 (1H, d, $J$ = 7.8 Hz, H1-Glc), 4.70 (1H, d, $J$ = 7.5 Hz H1-Gal), and 4.47 (1H, d, $J$ = 0.9 Hz H1-Rha)]. Furthermore, 31 carbon signals were observed in the $^{13}$C NMR spectrum. Among them, 13 carbon signals were assigned to the kaempferol and three carbon signals [$\delta$ 104.48 ($C_{Gal}$-1), 101.12 ($C_{Glu}$-1), and 102.21 ($C_{Rha}$-1)] were assigned to anomeric carbons of the sugar moiety. Compound **2** was also obtained as a pale yellow amorphous powder. Acid hydrolysis of **2** gave kaempferol, glucose, rhamnose, and xylose. The $^{1}$H NMR spectrum showed four kinds of aromatic protons [$\delta$ 8.00 (2H, d, $J$ = 8.7 Hz, H-2′, H-6′), 6.80 (2H, d, $J$ = 9.0 Hz, H-3′, H-5′), 6.34 (1H, d, $J$ = 2.1 Hz, H-8), and 6.14 (1H, d, $J$ = 2.1 Hz, H-6)] indicating the kaempferol skeleton with three anomeric protons [$\delta$ 5.35 (1H, d, $J$ = 7.2 Hz, H1-Glc), 4.73 (1H, d, $J$ = 7.5 Hz H1-Xyl), and 4.45 (1H, d, $J$ = 1.2 Hz H1-Rha)]. In addition, 30 carbon signals were observed in the $^{13}$C NMR spectrum. Among them, 13 carbon signals were assigned to the kaempferol and three carbon signals [$\delta$ 105.13 ($C_{Xyl}$-1), 100.83 ($C_{Glu}$-1), and 102.14 ($C_{Rha}$-1)] were due to the anomeric carbons of the sugar moiety. On the basis of the analysis data, compounds **1** and **2** were thus identified as kaempferol-3-*O*-[2-*O*-$\beta$-D-galactopyranosyl-6-*O*-$\alpha$-L-rhanmopyranosyl]-$\beta$-D-glucopyranoside and kaempferol-3-*O*-[2-*O*-$\beta$-D-xylopyranosyl-6-*O*-$\alpha$-L-rhamnopyranosyl]-$\beta$-D-glucopyranoside, respectively. All of these data corresponded to those in the literature for camelliaside A and camelliaside B, respectively (*7*). Although these compounds have already been reported as constituents in "tea seed cake" prepared from tea seeds, because tea seed cake is mixed material usually produced by tea seed, which are fruits born from a tea plant belonging to the *Camellia* family, this is the first report on the isolation of these compounds from natural GTSs, *C. sinensis* solely.

**Kaempferol Production from GTSE Using Glycolytic Enzymes.** Because conventional chemical hydrolysis inevitably produced side reactions, glycolytic enzymes have traditionally been used for the process of deglycosylation of products (*8*).

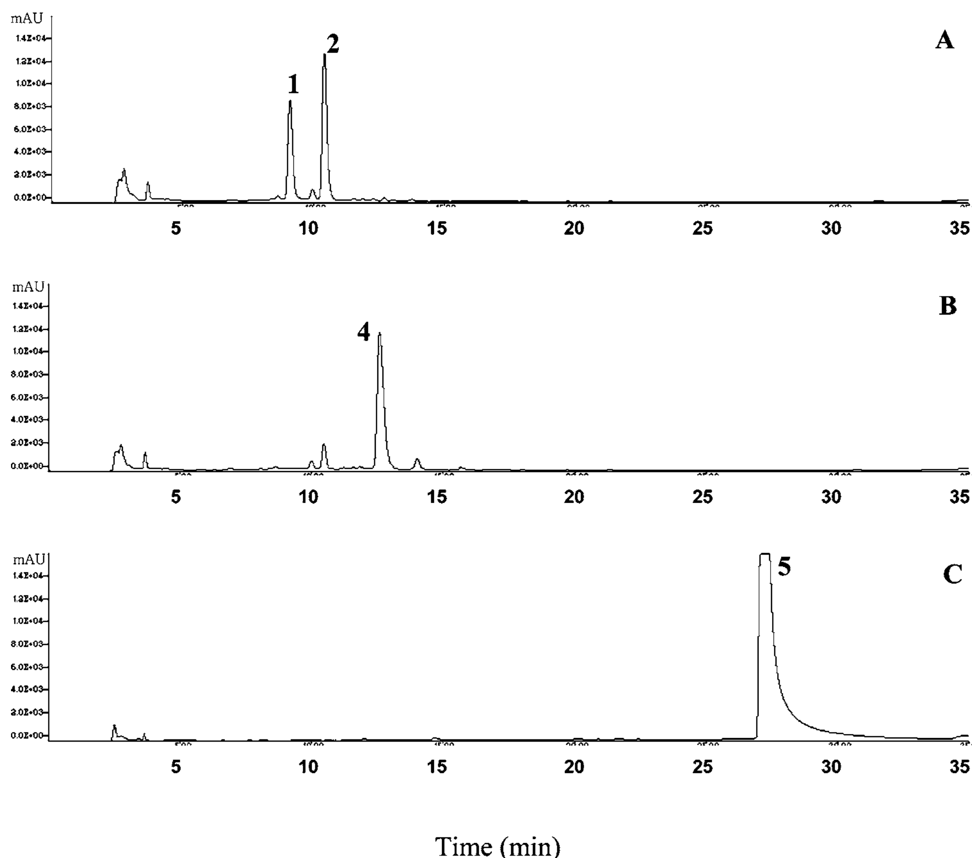
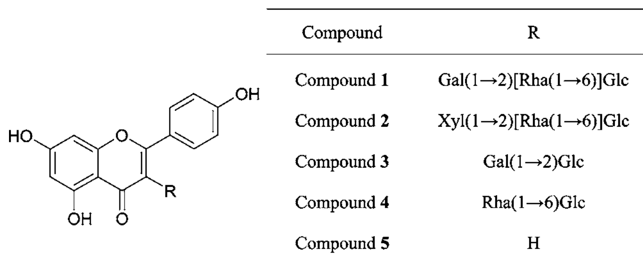



**Figure 2.** HPLC profile of hydrolysate of GTSE using mixed enzyme reaction. (**A**) GTSE, (**B**) reaction with $\beta$-galactosidase, (**C**) reaction with (**D**) and hesperidinase. Key to peak identity: 1, compound **1**; 2, compound **2**; 4, compound **4**; and 5, compound **5** (kaempferol).

To select enzymes for the concurrent bioconversion of kaempferol glycosides in GTS to kaempferol, the hydrolyzing ability of several glycolytic enzymes based on the glycosidic moiety of compounds **1** and **2**, hesperidinase, $\beta$-glucosidase, cellulase, $\beta$-glucuronidase, pectinase, $\beta$-galactosidase, amyloglucosidase, $\alpha$-amylase, $\beta$-xylosidase, xylanase, and Dextranase was evaluated. For this purpose, individual kinetics of hydrolysis of 50 mg/mL GTSE were carried out incubating each glycolytic enzyme at 37 °C for 24 h. The enzyme amount was adjusted to 50 units/g of GTSE. All reactions were monitored by HPLC and TLC analysis. Although some hydrolyzed compounds were produced on reaction with cellulase, amyloglucosidase, $\beta$-galactosidase, $\beta$-xylosidase, xylanase, and hesperidinase, complete hydrolysis to kaempferol was not achieved in any case (**Figure 1**). In the case of the reaction with hesperidinase (**Figure 1B**), kaempferol-3-*O*-$\beta$-D-galactopyranosyl (1→2)-$\beta$-D-glucopyranoside (compound **3**, peak 3) was produced by hydrolyzing the rhamnosyl moiety of compound **1**. The reaction with $\beta$-xylosidase, xylanase, cellulase, and amyloglucosidase gave the same hydrolysis pattern. **Figure 1C** shows the result of hydrolysis using cellulase. Kaempferol-3-*O*-$\beta$-D-rhamnopyranosyl (1→6)-$\beta$-D-glucopyranoside (compound **4**, peak 4) was produced by hydrolyzing the xylosyl moiety of compound **2**. In general, cellulase and amyloglucosidase were cleaved glycosidic bonds from some natural glycosides such as daidzin and genistin. However, in these results, they showed xylosyl bond cleaved activity, perhaps due to the different activities and longer incubation time. In the case of the reaction with $\beta$-galactosidase (**Figure 1D**), compound **4** was also produced by hydrolyzing the galactosyl moiety of compound **1** and the xylosyl moiety of compound **2**. However, kaempferol was not formed in any reaction. Furthermore, other glycolytic enzymes except mentioned above, $\beta$-glucosidase, $\beta$-glucuronidase, pectinase, $\alpha$-amylase, and dextranase, did not form any hydrolysis product. Although the flavonoids with glucose at the C-3 and C-7 positions can be a substrate for $\beta$-glucosidase, the deglycosylation of other sugar such as rhamnose and rutinose depends on the structure of the flavonoids, the position of the sugar substituent, and the species of the sugar moieties. We therefore carried out a novel enzymatic approach, which is the mixed enzyme reaction method, to complete deglycosylation of compounds **1** and **2** in GTSE simultaneously for kaempferol production. After various enzyme combination reactions according to their individual kinetic behavior, we were able to observe the optimum combination of the enzymes, hesperidinase and $\beta$-galactosidase. **Figure 2** shows the results of GTSE hydrolyzed with hesperidinase and $\beta$-galactosidase. When two enzymes were mixed for the reaction, compounds **1** and **2** were converted to kaempferol (peak 5) completely. This means that bioconversion of compounds **1** and **2** to kaempferol requires two enzyme reactions. Thus, $\beta$-galactosidase hydrolyzes the xylosyl and galactosyl moiety of compounds **1** and **2** and the following reaction with hesperidinase removes the remaining rhamnosyl-glucoside residue. **Figure 3** shows the structures of compounds **1** and **2** and their enzymatic hydrolysis products, compounds **3**−**5**.

**DPPH Scavenging Activities of Two Tea Seed Flavonoids and Kaempferol.** Most of the beneficial health effects of flavonoids are attributed to their antioxidant and chelating abilities. For comparing the antioxidation activity of tea seed flavonoids and its aglycone, kaempferol, the GTSE hydrolysate (GTSE-H) was further purified because the kaempferol content of GTSE-H was about 20% (w/w). After simple purification, centrifugation of GTSE-H and re-extraction of precipitation, we obtained the highly purified kaempferol in above 95% purity. The free radical scavenging activity of tea seed flavonoids and



| Compound | R |
|---|---|
| Compound **1** | Gal(1→2)[Rha(1→6)]Glc |
| Compound **2** | Xyl(1→2)[Rha(1→6)]Glc |
| Compound **3** | Gal(1→2)Glc |
| Compound **4** | Rha(1→6)Glc |
| Compound **5** | H |

**Figure 3.** Structure of glycosidic flavonoids isolated from GTS and its enzymatic hydrolyzed product, kaempferol. Key: Glc, glucopyranoside; Gal, galactopyranoside; Xyl, xylopyranoside; and Rha, rhamnopyranoside.

**Table 1.** DPPH Radical Scavenging Activity of Tea Seed Flavonoids and Kaempferol

| samples | $IC_{50}$ ($\mu$M)[a] |
|---|---|
| compound **1** | >1000 |
| compound **2** | >1000 |
| compound **5** (kaempferol) | 59.2 ± 1.7 |
| trolox | 51.3 ± 2.1 |
| L-ascorbic acid | 48.8 ± 2.7 |

[a] $IC_{50}$ denotes the antioxidant concentration causing 50% reduction of the free radical form of DPPH in methanol after 30 min with each sample, respectively. It was calculated from a regression line using different concentrations in triplicate experiments.

kaempferol, its aglycone, was tested by DPPH radical scavenging assay. The DPPH test is a nonenzymatic method currently used to provide basic information on the reactivity of compounds to scavenge free radicals (*9*). **Table 1** shows the DPPH radical scavenging activity of the two tea seed flavonoids, compounds **1** and **2**, and compound **5**, its aglycone. Compound **5**, kaempferol, inhibited DPPH formation by 50% at a concentration of 59.2 ± 1.7 $\mu$M ($IC_{50}$), whereas the two tea seed glycosides showed weak DPPH radical scavenging activity. Flavonoids generally occur in foods as *O*-glycosides with bound sugars, usually at the C-3 position. Recently, it has been reported that the aglycone is likely to have a greater biological effect than the glycoside (*9*). *O*-Glycosylation at C-3 of compounds **1** and **2** had a negative influence on the radical scavenging activity. The presence of a large substituent at C-3 reduced the activity, probably the steric hindrance.

**XO Inhibition Activities of Two Tea Seed Flavonoids and Kaempferol.** The enzyme XO catalyzes the oxidation of xanthine to uric acid. During the reoxidation of XO, oxygen molecules act as electron acceptors, producing superoxide radicals and hydrogen peroxide. Consequently, XO is considered to be an important biological source of superoxide radicals. These are involved in many pathological processes such as inflammation, cancer, and aging (*10*−*12*). To compare the anti-superoxide effect of tea seed flavonoids and kaempferol, both the inhibition of XO and the scavenging effect on the superoxide anion were measured in one assay. For comparison of inhibition effect of these compounds, allopurinol, the most well-studied xanthine oxidase inhibitor, was used as a positive control. Inhibition of XO results in a decreased production of uric acid, which can be measured specrophotometically, and a decreased production of superoxide was measured by the nitrite method (**Figure 4**). For each compound tested, two $IC_{50}$ values can be calculated by linear regression analysis: 50% inhibition of XO, which is calculated by a 50% decrease of uric acid production and a 50% reduction of the superoxide level. Each $IC_{50}$ value of the compound is listed in **Table 2**. Although all of the compounds showed less activity than allopurinol, the compound

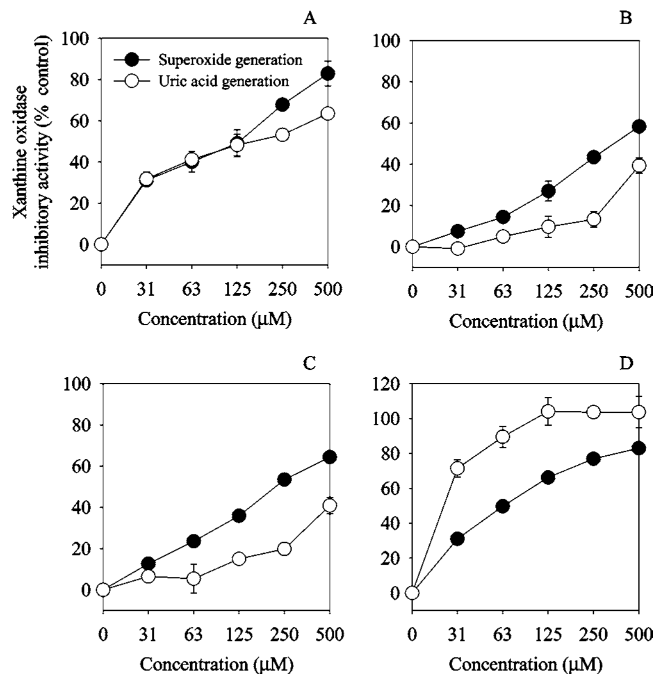



**Figure 4.** Antioxidation effect of tea seed flavonoids and kaempferol on xanthine/xanthine oxidase system. (**A**) Compound **5** (kaempferol), (**B**) compound **1**, (**C**) compound **2**, and (**D**) allopurinol. Open circles indicate the uric acid generation activity, and closed circles indicate the superoxide generation activity.

**Table 2.** Xanthine Oxidase Inhibitory Activity of Tea Seed Flavonoids and Kaempferol

| | xanthine oxidase inhibitory activity ($IC_{50}$,[a] $\mu$M) | |
|---|---|---|
| samples | superoxide generation inhibition | uric acid generation inhibition |
| compound **1** | 337.7 ± 18.0 | 660.2 ± 6.1 |
| compound **2** | 223.4 ± 3.3 | 386.9 ± 5.9 |
| compound **5** (kaempferol) | 108.7 ± 9.4 | 159.1 ± 4.9 |
| allopurinol | 63.1 ± 1.2 | 19.2 ± 3.2 |

[a] For each test, two $IC_{50}$ values were calculated by linear regression analysis: 50% inhibition of xanthine oxidase (=50% decrease of uric acid production) and 50% reduction of the superoxide level.

**5**, kaempferol, showed higher activity than its *O*-glycosides, compounds **1** and **2**. The presence of an OH group in 5- and 7-positions in both of the compounds makes them potential candidates for superoxide reaction (*13*). Free radical scavenging by flavonoids is highly dependent on the presence of a free 3-OH (*14*). Usually, aglycones are more potent antioxidants that their corresponding glycosides (*15*). In this case, our result confirmed that *O*-glycosylation at C-3 reduced the activity of superoxide reaction although the kaempferol glycosides have a free OH group in both 5- and 7-positions.

Two kaempferol glycosides, kaempferol-3-*O*-[2-*O*-$\beta$-D-galactopyranosyl-6-*O*-$\alpha$-L-rhamnopyranosyl]-$\beta$-D-glucopyranoside and kaempferol-3-*O*-[2-*O*-$\beta$-D-xylopyranosyl-6-*O*-$\alpha$-L-rhamnopyranosyl]-$\beta$-D-glucopyranoside, were isolated from the GTS. From these compounds, a commercially and biologically useful flavonoid, kaempferol, was produced by enzymatic hydrolysis using two O-glycolytic enzymes, $\beta$-galactosidase and hesperidinase. After a simple separation process, we obtained a highly purified kaempferol. We also compared the antioxidant effect of these two GTS flavonoids and its aglycone, kaempferol.

Kaempferol is a more efficient scavenger of DPPH radicals and a better inhibitor of xanthine/xanthine oxidase than the two glycosides.

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