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Isolation of 3-Galloylprocyanidin B_3, a Glucosyltransferase Inhibitor from the Korean Green Tea Leaves

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In the course of surveying the anti-plaque agents for dental caries prevention, the extract of Korean green tea leaves (KGTL) was tested for inhibitory activity against *Streptococcus mutans* adhering to glass surfaces in the presence of crude glucosyltransferase (GTase). The extracts of KGTL showed a positive inhibitory activity against GTase. The active compound was purified through Sephadex LH-20 and MCI gel CHP-20P columns. A positive reaction was shown in the anisaldehyde-H_2SO_4 test, which confirmed the condensed tannin. The inhibitory compound was identified as 3-galloylprocyanidin B_3 through IR, negative FAB-mass, and ^{1}H-NMR spectroscopic analyses. Acetone extract and 3-galloylprocyanidin B_3 of KGTL showed inhibitory effect against GTase. The percent of inhibition was determinated to be 71.84% ($P<0.01$) with 10 mM 3-galloylprocyanidin B_3. The 3-galloylprocyanidin B_3, which possessed a galloyl, showed a higher inhibitory activity against glucosyltransferase than monomeric (+)-catechin and procyanidine B_3 which had no galloyl group.

Key words: *green tea, 3-galloylprocyanidin B_3, glucosyltrasferase inhibitor.*

Tannins are water soluble phenolic compounds which occur widely in vascular plants[1,2] and are known to have adverse effects on such diverse organisms as viruses, bacteria, fungi, insects, reptiles, birds, and mammals.[2] They have been accorded an important role in protecting plant tissues from attack of herbivore,[3,4] although Bernays[5] has recently stressed the variability of the effects of tannins on insect herbiviores and Bate and Metcalfe[6] has cautioned against premature generalizations concerning their evolutionary and ecological significances.

Since dental caries was found to be one of the infectious diseases caused primarily by *Streptococci* in the oral flora, many studies have been performed on GTase from *Streptococcus mutans*, the primary bacterium causing dental caries, in experimental animals.[7,8] The bacterium produces water-soluble and -insoluble glucans from sucrose through the cell-bound or extracellular glucosyltransferase. The sticky insoluble glucan facilitates the accumulation of microorganisms on the smooth tooth surface to form dental plaque and the subsequent development of dental caries.[9,10] As a study on anti-plaque substances, active ingredients which exhibit inhibitory activities against the adherence of *S. mutans* cells to smooth surfaces was investigated.[11-13] In this study, to utilize natural inhibitor against the glucan forming reaction by GTase from *S. mutans*, a GTase-inhibiting compound has been purified from the KGTL. The active compound was identified through the structure analyses, and the inhibitory effect against GTase was determined.

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Abbreviations: GTase, glucosyltransferase; KGTL, Korean green tea leaves; TLC, thin layer chromatography.

Materials and Methods

Material and reagents. KGTL (1 kg) was purchased at a local store in May 1999. For isolation, Sephadex LH-20 (Pharmacia Co., Sweden) and MCI gel CHP-20P (Mitsubishi Co., Japan) were used. All other chemicals used in this study were of analytical grade. Melting point was measured using a micro-melting pointer (Shimadzu, Chiyoda, Japan), and $[\alpha]_D$ was measured with a polarimeter (Jasco, DI-370, Japan). For analysis of ^{1}H-NMR (Jeol, GX-270, Japan), chemical compounds were dissolved in acetone-d_6-D_2O. TMS was used as a standard material. Molecular weight was measured with a negative ion FAB-MS (Jeol, JMX-DX-300, Japan).

Extraction and isolation. Per 1 kg of dried KGTL, 2 *l* of 60% acetone was added and soaked for 24 h. After being centrifuged at 8000 rpm for 20 min, the supernatant was separated. The procedure was repeated three times to obtain the supernatant which was then concentrated with a vacuum evaporator and used as the experimental material for purification. Purification was done using Nonaka's method.[14] The 60% acetone extract of KGTL was concentrated into 0.5 *l*, loaded on to a Sephadex LH-20 column (5×120 cm), and eluted using a gradient ethanol (0→40%). Isolated fractions were loaded on an MCI gel CHP-20P column (3×45 cm) and isolated using the gradient method of Nonaka (0→100% MeOH). Eluate was identified through the reaction color with anisaldehyde and R_f value on the silica gel TLC. Isolated tannin was lyophilized. The result of column chromatography, fractions A and B, and

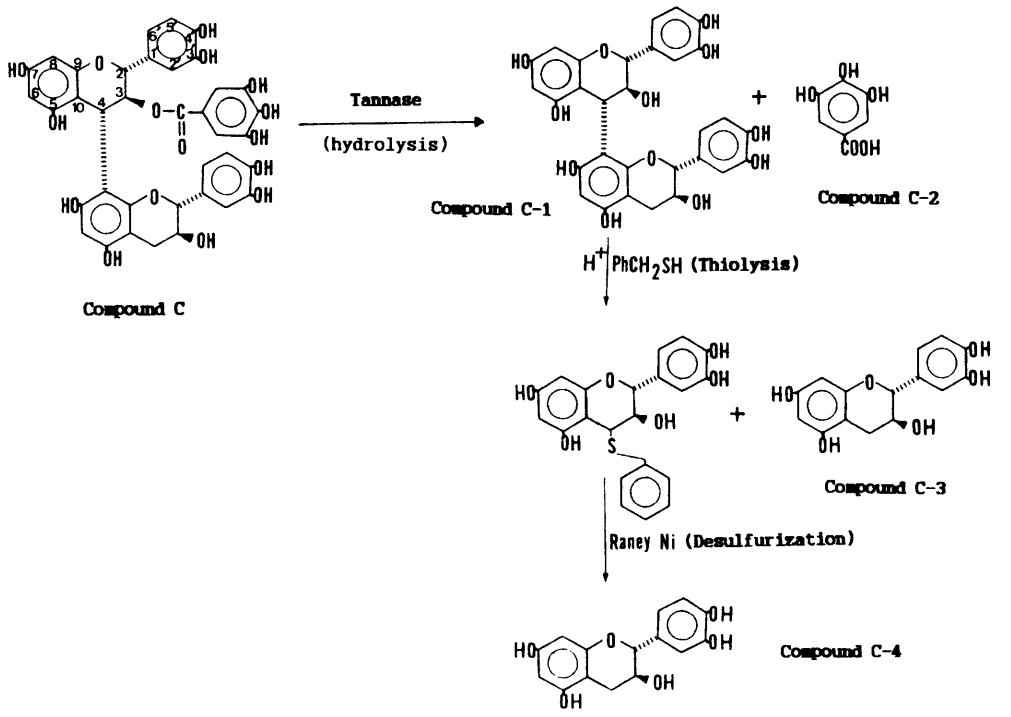

Fig. 1. The procedure of hydrolysis, thiolysis and desulfurization of compound C.

compound C were separated from KGTL.

Compound C: amorphous powder of dark orange color; $[\alpha]_D^{21}$ -216.1 °(c = 0.98, acetone); IR ν_{max} 1710 (COO), 1600 (C = C)cm^{-1}, ^{1}H-NMR (acetone-d_6-D_2O) 2.57~2.59 (2H, m, H-4'), 3.23~3.74 (2H, m, H-3,3'), 4.57 (1H, brs, H-4), 4.97~5.00 (2H, m, H-2,2'), 6.25~6.50 (3H, m, H-A,A'ring), 6.90~7.28 (6H, m, H-B,B'ring), 7.71 (2H, each s, H-galloyl); FAB-MS (negative ion mode) *m/z* 729[M-H]$^-$.

Derivation of compound C. Following Matsuo and Ito's method,[15] 120 mg of the compound C that was purified as single compound among separated fractions A, B, and C was resolved in 2.5 m*l* of sodium phosphate buffer (pH 6.6), and tannase was added for hydrolysis of gallate. The solution was reacted at 30°C for 2 h (Fig. 1). After 50 mg of the compound C-1 was resolved in 5 ml of 5% acetic acid in ethanol and 2 m*l* of benzylmercaptan was added to the solution, thiolysis reaction was done at 100°C. The reaction solution was separated on TLC using benzene : ethylformic acid : formic acid (1 : 7 : 2), and purified through Sephadex LH-20 and MCI gel CHP-20P column chromatographies using 60% methanol and ethanol, respectively. Then, flow rate was 1.8 m*l*/min. The obtained compound was used for structural analysis (Fig. 1). Fifty milligrams of the compound was resolved in 1 m*l* of acetic acid : ethanol (1 : 9). One hundred milligrams of Raney nickel was added to this solution, stood for 1 h at room temperature for desulfurization, and filtered to remove the Raney nickel. The reacted product was purified through Sephadex LH-20 and MCI gel CHP-20P column chromatographies using 60% methanol and ethanol, respectively. The result of column chromatography, derivative of C-1, C-2, C-3 and C-4 from purified compound C were obtained (Fig. 1).

Compound C-1: amorphous powder of dark brown color, $[\alpha]_D^{25}$ -198.1°(c = 1.0, acetone); ^{1}H-NMR (acetone-d_6-D_2O) δ 2.53~3.24 (2H, m, H-4'), 3.85~5.09 (5H, m, H-2,2',3,3',4), 5.78~6.21 (3H, m, H-6,8,6'), 6.37~7.02 (6H, m, H-B,B'ring).

Compound C-2: Colorless needles, mp; 266~268°C, IR ν_{max} 3400 (OH), 1710 (COO)cm^{-1}, ^{1}H-NMR (acetone-d_6-D_2O) δ 7.14 (2H, each s, H-galloyl).

Thiolized compound: amorphous powder of dark brown color, $[\alpha]_D^{25}$+25.4° (c = 0.7, acetone); ^{1}H-NMR (acetone-d_6-D_2O) δ 4.09 (2H, s, -SCH_2-), 4.00~4.24 (1H, m, H-3), 4.38 (1H, d, J = 4 Hz, H-4), 4.94 (1H, d, J = 10 Hz, H-2), 5.82 (1H, d, J =2 Hz, H-6), 6.03 (1H, d, J = 2 Hz, H-8), 6.76 (1H, dd, J = 8 Hz, 2 Hz, H-6'), 6.84 (1H, d, J = 8 Hz, H-5'), 6.93 (1H, brs, H-2'), 7.167.46 (5H, m, H-aromatic)

Compound C-3: Colorless needles, mp; 172~175°C; $[\alpha]_D^{25}$+9.4°(c = 1.0, acetone); IR ν_{max} 3440(OH), 1620 (aromatic, C = C) cm^{-1}, ^{1}H-NMR (acetone-d_6-D_2O) δ 2.57 (1H, dd, J = 16 Hz, 8 Hz, H-4), 2.89 (1H, dd, J = 16 Hz, 6 Hz, H-4), 4.02 (1H, m, H-3), 4.60 (1H, d, J = 8 Hz, H-2), 5.93 (1H, d, J = 2 Hz, H-6), 6.03 (1H, d, J = 2 Hz, H-8), 6.79 (1H, dd, J = 8 Hz, 2 Hz, H-6'), 6.83 (1H, d, J = 8 Hz, H-5'), 6.92 (1H, d, J = 2 Hz, H-2'); FAB-MS (negative ion mode) *m/z* 289[M-H]$^-$.

Compound C-4: The same with compound C-3.

Thin layer chromatography. The compounds isolated from the gels were developed using silica gel TLC (5×5 cm) with benzene : ethylformic acid : formic acid (1 : 7 : 2)

Table 1. Inhibitory effects of acetone extract from Korean green tea on glucosyltransferase activity.

Concentration (mg/ml)	Absorbance (550 nm)	Inhibition (%)
Control	1.095±0.016	---
1.0	0.599±0.014**	45.30**
2.0	0.518±0.011**	52.69**

**P<0.01, *P<0.05

Table 2. Inhibitory effects of 3-galloylprocyanidin B_3 concentration on glucosyltransferase activity.

Concentration (mM)	Absorbance (550 nm)	Inhibition (%)
Control	0.870±0.016	---
0.1	0.878±0.024	---
0.5	0.801±0.027	7.93
1.0	0.595±0.018*	31.61*
2.0	0.527±0.005**	39.43**
5.0	0.403±0.009**	53.68**
10.0	0.245±0.014**	71.84**

**P<0.01, *P<0.05

through Matsuo and Ito's method.[15] For identification, the compounds were reacted with anisaldehyde-H_2SO_4 (ethanol : H_2SO_4 : anisaldehyde = 18 : 1 : 1) and $FeCl_3$ (1% in methanol) on silica gel TLC.[15] The structures, developed types, and colors of the reaction materials were examined.

Determination of inhibition against GTase. Inhibition rate of GTase, prepared according to Nam *et al.*[16], was 6 times repeatedly measured using the method of Endo *et al.*[17] Sucrose was used as a substrate, and the glucan produced was measured with a spectrophotometer. Purely refined extracts were made in various concentrations and resolved in 0.18 m*l* of sodium phosphate buffer (pH 6.8) solution. Subsequently, 0.8 m*l* of the substrate (sucrose +NaN_3) and 0.02 m*l* of GTase were added to the solution and mixed well in a glass tube. Reaction was done at 37°C for 16 h slanted at 30°. The solution was washed twice with 3 m*l* distilled water, and the soluble glucan was removed. Three milliliter distilled water was added to the reacted solution. The reactant was separated through sonication, and the absorbance measured at 550 nm.

Results and Discussion

Effect of acetone extracts of KGTL on GTase inhibition. Inhibitory effect of acetone extracts of KGTL was determined at concentrations of 1~2 mg. As shown Table 1, The percent of inhibition on GTase were about 45% (P<0.01) and 53% (P<0.01) at 1 and 2 mg, respectively.

Isolation of tannins. The tannin compounds suspected as the inhibitor of GTase were isolated using Nonaka's method,[14] purified through Sephadex LH-20 and MCI gel CHP-20P columns. Fraction A (3.84 g), fraction B (26.5 g), and fraction C (309.43 mg) were obtained. The result of the color reaction of these fractions on silica gel TLC showed the compounds have a flavan-3-ol skeleton as shown by the fact that they changed to red in anisaldehyde solution and to blue in $FeCl_3$ solution. This characteristics of the fractions were the same as those of the condensed tannin reported by Nonaka *et al.*[18] Fraction A and B were observed to be types of various mixed tannin compounds and Fraction C to be a single band through TLC. Thus fraction C was purified as a single compound through Sephadex LH-20 and MCI gel CHP-20P column chromatographies. The Rf value of purified compound C was about 0.1.

Determination of chemical structures of the purified compound. The chemical analysis data were obtained from the purified compound C. As shown Fig. 1, compound C-1 and C-2 from compound C by hydrolysis with tannase and compound C-3 and C-4 from compound C-1 by thiolysis and desulfurization were obtained at the same ratio. Compound C-2 was identified as gallic acid by ^{1}H-NMR signal type (singlet, galloyl-H) and the spectral characteristics of compound C-1 were assumed to procyanidin B_3[12,19]. As compound C-1 showed a complicated ^{1}H-NMR spectrum, compounds C-3 and C-4 were separated from compound C-1. Compounds C-3 and C-4 were identified (+)-catechin[15] by FAB-MS, IR and ^{1}H-NMR spectrum, respectively. Thus, Compound C was not only linked to (+)-catechin and (+)-catechin but also to gallic acid at C-3 of C-ring. This compound was characterized as 3-galloylprocyanidin B_3.

Effect of 3-galloylprocyanidin B_3 on GTase inhibition. Inhibitory effect of the purified 3-galloylprocyanidin B_3 on GTase was determined at concentrations of 0.1 to 10.0 mM. As shown Table 2, the percent of inhibition on GTase at 10 mM was determined to be 71.84% (P<0.01). The inhibitory effect of 3-galloylprocyanidin B_3 of KGTL on GTase was higher than the 56.12% (P<0.01) inhibitory effect at 10 mM of (+)-catechin and 68.07% (P<0.01) inhibitory effect at 10 mM of procyanidin B_3 from Jack fruit leaves reported by Ahn *et al.*[12] These results showed that the inhibition activity of the proanthocyanidin (procyanidin) system was higher than those of the monomeric flavan-3-ol compounds. Furthermore the inhibition rate of the compound containing the galloyl was higher than that with no galloyl compound. These were in accord with the result of Hayashi *et al.*[20] and Hattori *et al.*[21] reported that the compounds containing gallate highly inhibited the enzyme activity.

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