

Preparation, Characterization, and Antioxidative Effects of Oligomeric Proanthocyanidin–L-Cysteine Complexes

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Controlled acid-catalyzed degradation of proanthocyanidin polymers in grape seeds together with L-cysteine led to oligomeric proanthocyanidin–L-cysteine complexes along with monomeric flavan-3-ol derivatives being isolated, and their structures were confirmed on the basis of spectroscopic data and by chemical means. In addition, comparative studies on the antioxidative and survival effects of oligomeric proanthocyanidin–L-cysteine complexes and proanthocyanidin polymers were performed. The oligomeric proanthocyanidin–L-cysteine complexes showed higher bioavailability and antioxidant capacity and enhanced survival time in the animal test groups. In addition, it is suggested that the oligomeric complexes may help to prevent oxidative stress and may reduce free radical production.

KEYWORDS: Proanthocyanidin; oligomer; polymer; proanthocyanidin–L-cysteine complex; L-cysteine; polyphenol; flavan-3-ol; oligomerization; grape seed polyphenol; antioxidative activity; survival study

INTRODUCTION

Among plant polyphenols, proanthocyanidins are increasingly attracting attention for their antiaging effects and preventive properties in relation to the development of multiple human ailments, such as hypertension, allergies, infection, cardiovascular diseases, and a variety of infections (1–8). Naturally occurring proanthocyanidins are complicated mixtures, consisting primarily of flavan-3-ol polymers. They are, because of their large molecular sizes, regarded as not being readily absorbed through the intestines. On the other hand, it has been reported that radiolabeled monomeric flavan-3-ols and proanthocyanidin dimers and trimers can be readily transported through a layer of colonic carcinoma (Caco-2) cells of human origin (9). The dimer, procyanidin B-2, is also described as being readily detectable in human plasma after flavan-3-ol-rich cocoa has been consumed (10). Furthermore, proanthocyanidin oligomers have been reported to have much stronger bioactivities than flavan-3-ol monomers and polymers (11–15). Therefore, it would be beneficial to develop an available source of oligomers that could be easily taken up by cells.

Thiolysis is a known method in which the depolymerization of proanthocyanidins through a cleavage of interflavanoid linkages, followed by a nucleophilic attack by a thiol group on the C-4 position of a flavan moiety, enables the structures of

the flavan-3-ol units and the degree of polymerization to be confirmed (16). Benzylthiol or 2-hydroxyethylthiol has typically been used as a thiolytic reagent. It is, however, questionable as to whether such sulfur-containing products resulting from this process are suitable to be utilized as foods. As an alternative, monomeric flavan-3-ol–L-cysteine complexes were previously obtained by depolymerization of proanthocyanidin in the presence of L-cysteine by Torres et al. (17). L-Cysteine itself is safe for food supplements; thus, L-cysteine derivatives herein may be also safe for food application.

On the basis of this background, the purposes of this study were to establish the most suitable method to prepare proanthocyanidin oligomers from polymers through acid-catalyzed degradation in the presence of L-cysteine and to compare the antioxidative effects and rate of animal survival when using the oligomers with those of original polymers.

MATERIALS AND METHODS

Chemicals. L-Cysteine hydrochloride monohydrate and tannase (from *Aspergillus oryzae*) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). L-Ascorbic acid was obtained from Junsei Chemical Co., Ltd. (Tokyo, Japan). (+)-Catechin, (–)-epicatechin, (–)-epigallocatechin, (–)-epicatechin 3-O-gallate, and procyanidins B-1 and B-2 were obtained from Funakoshi Co., Ltd. (Tokyo, Japan). The Sephadex LH-20, DIAION HP-20, and MCI-gel CHP 20 that were used were produced by Amersham Biosciences K.K. (Tokyo, Japan) and Mitsubishi Chemical Corp. (Tokyo, Japan), respectively.

Analyses. ¹H (400 MHz) and ¹³C (100 MHz) nuclear magnetic resonance (NMR) spectra were recorded on a Jeol GX-400 using TMS or corresponding solvent signals as an internal standard at 25–40 °C.

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High-resolution fast atom bombardment mass spectra (HR-FAB-MS) and high-resolution electrospray ionization mass spectra (HR-ESI-MS) were obtained with a JEOL JMS-700TZ. Optical rotations were measured with a Jasco DIP-370 digital polarimeter at room temperature. Thin-layer chromatography (TLC) was performed on a precoated 0.25 mm Kieselgel 60 F₂₅₄ glass plate (Merck KGaA, Darmstadt, Germany) with benzene/ethyl formate/formic acid (2:7:1) and *n*-butanol/acetic acid/H₂O (4:1:2) and was detected by irradiation of UV light (254 nm) and by spraying 2% ethanolic FeCl₃, anisaldehyde–sulfuric acid, or 0.2% *n*-butanolic ninhydrin reagents.

High-Performance Liquid Chromatography (HPLC). Reverse-phase HPLC was performed using a Hitachi apparatus equipped with an L-7420 detector (at 260 nm) and a 150 × 4 mm i.d., 5 μm, ODS 80Ts column (Toso Co., Ltd., Tokyo, Japan). Separation was achieved with an increasing amount of 0.1% acetic acid in acetonitrile (B) in 0.1% aqueous acetic acid (A): 0–5 min, 5% B, isocratic; 5–20 min, 5–70% B, linear gradient; 20–20.1 min, 70–90% B, linear gradient; and 20.1–25 min, 90% B, isocratic at a flow rate of 0.8 mL/min. Normal-phase HPLC was achieved using the same apparatus with a 250 × 4.6 mm i.d., 5 μm, Luna Silica column (Phenomenex, Torrance, CA) at 40 °C, and was recorded at 280 nm. The mobile phases consisted of CHCl₃/MeOH/H₂O/0.5% trifluoroacetic acid (400:90:5:5) (A) and CHCl₃/MeOH/H₂O/0.5% trifluoroacetic acid (45:405:45:5) (B). Separation was achieved at a flow rate of 0.8 mL/min with the following gradient system: 0–20 min, 0–15% B, linear gradient; 20–45 min, 15–30% B, linear gradient; and 45–70 min, 30% B, isocratic.

Preparation of the Oligomeric Proanthocyanidin–L-Cysteine Complexes from Grape Seed. Dried grape seeds (8.0 kg) were powdered and extracted with 80% MeOH (30 L) at room temperature for 3 days. The insoluble materials were filtered out, and the filtrate was concentrated and applied to a column of DIAION HP-20 (ca. 25 L). After a washing with H₂O (100 L), elution with MeOH (50 L) gave a mixture of grape seed proanthocyanidins (GSP) as a dark brown powder (456 g). A mixture of GSP (400 g), L-cysteine hydrochloride monohydrate (400 g), and L-ascorbic acid (4 g) in H₂O (4 L) was kept with stirring at 60 °C for 48 h. The reaction mixture was subjected to a column of DIAION HP-20 (ca. 25 L). After a washing with H₂O (100 L), elution with 40% EtOH (50 L) yielded oligomeric proanthocyanidin–L-cysteine complexes (cys–OLG) as a reddish brown powder (408 g).

Separation of Cys–OLG. Cys–OLG (50 g) in H₂O (0.15 L) was partitioned with a mixture of *n*-butanol, *n*-propanol, and H₂O (2:1:3, 0.6 L) six times. The aqueous layer was concentrated and lyophilized to give a polar fraction (fraction I) as a brown powder (7.2 g), whereas the organic layer, after concentration, afforded an oligomeric fraction as a deep brown powder (42.5 g). A portion (15 g) of this powder was chromatographed over Sephadex LH-20 with an increasing polarity from EtOH to EtOH/H₂O (4:1) to separate fractions into II (1.37 g) and III (4.05 g). Repeated chromatography of fraction II (1 g) using Sephadex LH-20 and MCI gel CHP 20 with an increasing amount of MeOH in H₂O (9:1–1:9) gave compounds **1** (288.1 mg), **2** (14.5 mg), and **3** (48.2 mg), whereas fraction III (3 g) yielded compounds **4** (25.3 mg), **5** (16.2 mg), and **6** (43.4 mg) upon similar treatment. These compounds showed positive spots with a ninhydrin spray reagent on TLC.

Compound **1** was obtained as a white amorphous powder: [α]_D –62.7° (*c* 1.0, 50% acetone); HR-FAB-MS, *m/z* 410.0925 [M + H]⁺.

Compound **3** was obtained as a pale brown amorphous powder: [α]_D –64.1° (*c* 1.0, 50% acetone); HR-FAB-MS, *m/z* 426.0844 [M + H]⁺.

Compound **4** was obtained as a pale brown amorphous powder: [α]_D +44.0° (*c* 1.0, methanol); negative HR-ESI-MS, *m/z* 696.1360 [M – H][–].

Compound **5** was obtained as a pale brown amorphous powder: [α]_D +1.0° (*c* 0.2, 50% acetone); negative HR-ESI-MS, *m/z* 848.1486 [M – H][–].

Compound **6** was obtained as a brown amorphous powder: [α]_D +56.2° (*c* 1.0, 50% acetone); negative HR-ESI-MS, *m/z* 984.1956 [M – H][–].

Enzymatic Hydrolysis. A solution of compound **5** (18 mg) in H₂O (4.5 mL) was incubated for 3 h with tannase (3.6 mg) at 37 °C. The solvent was evaporated under reduced pressure, and the residue was treated with MeOH. The soluble portion of MeOH was chromatographed over Sephadex LH-20 (ca. 20 mL) with 70% EtOH to give gallic acid (3 mg) and hydrolysate (**4**, 11 mg).

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Animal Studies. Animals and Treatment. The “Guidelines for Animal Experimentation” approved by Amino Up Chemical Co., Ltd. were followed in these experiments. Specific pathogen-free (SPF) male 9-week-old Wistar rats were purchased from Japan SLC, Inc. (Shizuoka, Japan) to evaluate the antioxidant capacity, and SPF male 5-week-old ddY mice were purchased from Japan Laboratory Animals, Inc. (Tokyo, Japan) for the survival study. They were housed under environmentally controlled conditions at a temperature of about 23 °C, a relative humidity of about 55%, and a 12-h alternating cycle of light and dark. All rats and mice were allowed access to a solid diet (CE-2, comprising 24.0% protein, 3.5% lipid, and 60.5% carbohydrate) (CLEA Japan Inc., Tokyo, Japan) and drinking water ad libitum. Following 1 week of adaptation, 35 10-week-old rats were randomly divided into seven groups (5 animals per group), which were control (no treatment), GSP, and cys–OLG groups for experiment 1 (expt 1) and control (no treatment), fraction I, fraction II, and fraction III groups for experiment 2 (expt 2). Both experiments were independently carried out. Each material was administered daily by oral gavage at a dose of 10 mg/kg of body weight for 1 week. Blood was taken from the jugular vein before the initial administration and 24 h after the last administration. Serum was immediately separated from the blood samples by centrifugation at 3000 rpm for 10 min. In the experiment of survival study on normal mice, 36 6-week-old mice were randomly divided into three groups (12 animals per group), which were control (normal powder diet, CE-2), GSP, and cys–OLG groups. Each group was daily given a powder diet containing each material at a dose of 200 mg/kg of body weight. After 6 months, blood was obtained from five fasted mice of each group for antioxidant evaluation following anesthesia by diethyl ether, and serum was prepared by centrifugation at 3000 rpm for 10 min. The remaining mice were continuously treated in the same manner until seven control animals all died naturally.

Analyses. The polyphenol concentration was measured using a method based on that of Gulcin (18). One hundred and twenty microliters of serum was mixed with 40 μL of 60% perchloric acid and extracted with 600 μL of *n*-butanol for 10 s. After centrifugation at 12000 rpm for 10 min at 4 °C, a 200-μL aliquot of the supernatant fraction was dried under nitrogen gas purge for 15 min at 40 °C. Then, 1.0 mL of 0.1 M FeCl₃ was added, and the reaction was initiated by adding 80 μL of 10 mM K₃[Fe(CN)₆]. After 20 min of incubation at room temperature, color intensity was measured at 720 nm. The levels of Trolox equivalent antioxidant capacity (TEAC) and lipid peroxide (LPO) were evaluated as parameters of antioxidant capacity. For the TEAC assay (19), 300 μL of 500 μmol/L 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 36 μL of metmyoglobin, 487 μL of 5 mM phosphate buffer (pH 7.4), and 10 μL of sample were mixed, and the reaction was initiated by the addition of 167 μL of 450 μmol/L hydrogen peroxide. The absorbance at 734 nm was measured after being incubated for 15 min at room temperature. LPO concentrations were evaluated using a LPO-Test Wako assay kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Statistics. Antioxidant capacity data are presented as mean ± SD and were analyzed by one-way ANOVA. Fisher's protected least significant difference (PLSD) was used as post hoc test. Regarding the survival study, survival rates were assessed using the Kaplan–Meier method, and differences between the rates were analyzed by the log-rank test. Significance was considered at *p* < 0.05.

RESULTS AND DISCUSSION

Preparation of Cys–OLG. The ratio of cysteine moiety in cys–OLG was estimated by microanalysis and displayed 2.70% sulfur content. Considering the molecular weights of sulfur (32.1), cysteine (121.16; C₃H₇NO₂S), and catechin (290.27), the ratio of cysteine and catechin units is calculated as 1:3.67.

Characterization of Compounds. Compound **1** showed an [M + H]⁺ peak in the HR-FAB-MS, which was in agreement with the molecular formula of C₁₈H₂₀O₈NS. The ¹H NMR data for **1** (Table 1) showed that it was closely analogous to (–)

Table 1. ¹H Chemical Shifts for 1–5

H no.	1 ^a	2 ^b	3 ^b	4 ^a	5 ^b
H-2	5.09 (br s)	4.86 (d, 9.6 Hz)	5.13 (s)	4.96 (br s)	5.3
H-3	4.07 (m)	4.22 (dd, 9.6, 4.3 Hz)	4.07 (s)	4.22 (br m)	5.4
H-4	3.86 (d, 2 Hz)	4.23 (d, 4.3 Hz)	3.92 (s)	4.54 (br s)	4.6
H-6	5.99 (d, 2 Hz)	6.04 (d, 2 Hz)	6.03 (br s)	5.92	5.9 or 6.1
H-8	5.87 (d, 2 Hz)	5.81 (d, 2 Hz)	5.96 (br s)	5.92	5.9 or 6.1
B-H-2	6.99 (d, 2 Hz)	6.94 (br s)	6.57 (s)	6.60–7.09	6.5–6.75
B-H-5	6.76 (d, 8.3 Hz)	6.86 (br s)		6.60–7.09	6.5–6.75
B-H-6	6.81 (dd, 8.3, 2 Hz)	6.86 (br s)	6.57 (s)	6.60–7.09	6.5–6.75
H-2'				5.20 (br s)	5.3
H-3'				3.90 (s)	4.0
H-4'				3.83 (s)	4.0
H-6'				5.92	5.9 or 6.1
B'-H-2				6.60–7.09	6.5–6.75
B'-H-5				6.67 (d, 8.3 Hz)	6.5–6.75
B'-H-6				6.60–7.09	6.5–6.75
gal-H-2					6.8 (br m)
gal-H-6					6.8 (br m)
cys-H-2	4.13 (dd, 9, 4 Hz)	4.07 (dd, 9, 4 Hz)	4.10 (br m)	4.14 (dd, 9, 4 Hz)	4.07 (dd, 9, 3.9 Hz)
cys-H-3	2.95 (dd, 15, 9 Hz)	3.02 (dd, 15, 9 Hz)	3.00 (dd, 15, 9 Hz)	2.95 (br m)	3.0 (br m)
	3.43 (dd, 15, 4 Hz)	3.55 (dd, 15, 4 Hz)	3.45 (dd, 15, 4 Hz)	3.44 (br m)	3.6 (br m)

^a Measured in acetone-*d*₆-D₂O. ^b Measured in CD₃OD at 25 °C.

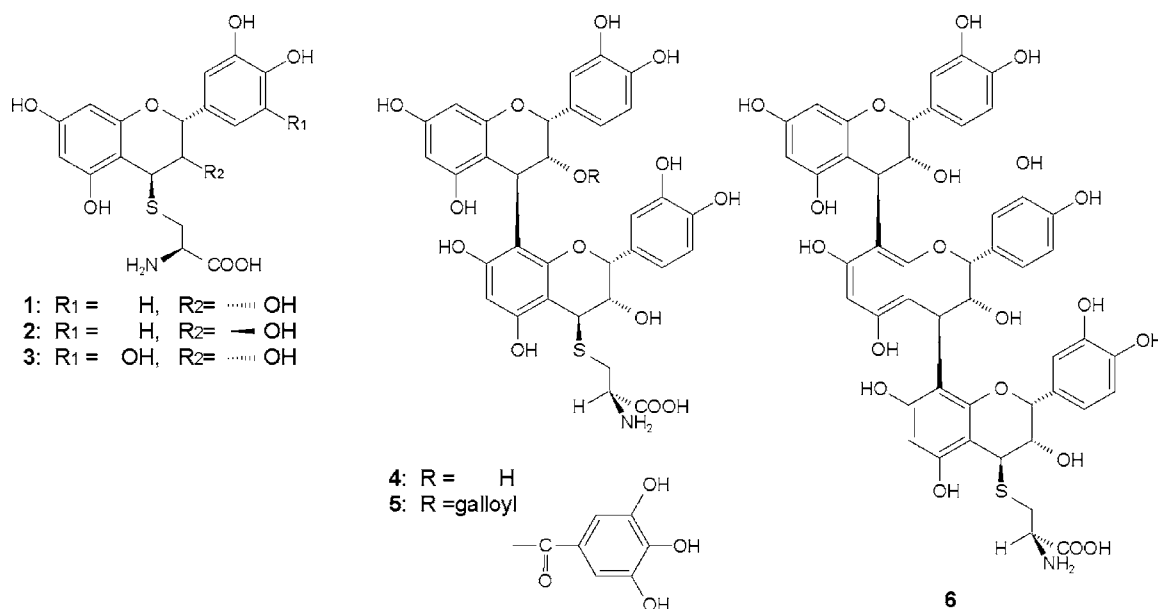


Figure 1. Structures of compounds 1–6.

epicatechin (2,3-*cis* orientation) as it displayed a broad singlet signal at δ 5.09 due to H-2. This was supported by the ¹³C NMR chemical shift (δ 74.7) due to the epicatechin C-2 carbon (20). In the ¹H NMR spectra, the appearance of a set of aliphatic signals at δ 5.09 (1H, s), 4.07 (1H, m), and 3.86 (1H, d, J = 2 Hz), assignable to flavan H-2, -3, and -4, respectively, indicated the presence of a 4-substituted flavan ring with 2,3-*cis* and 3,4-*trans* configurations in the C-ring (21). In addition, the appearance of ABX signals at δ 4.13 (1H, dd, J = 9, 4 Hz), 2.95 (1H, dd, J = 15, 9 Hz), and 3.43 (1H, dd, J = 15, 4 Hz) suggested the presence of a cysteine moiety. On the basis of these findings, the structure of **1** was deduced as 4 β -(*S*-L-cysteinyl)-(-)-epicatechin as shown in **Figure 1**. These spectroscopic data are identical with those reported by Torres et al. (17).

The ¹H NMR spectrum of **2** (**Table 1**) was similar to that of **1**, except for the signals due to flavan H-2, -3, and -4 at δ 4.86 (1H, d, J = 9.6 Hz), 4.22 (1H, dd, J = 9.6, 4.3 Hz), and 4.23 (1H, d, J = 4.3 Hz), respectively, the larger coupling constants suggesting the 2,3-*trans* and 3,4-*trans* configurations (20). Thus,

the structure of **2** was concluded to be 4 β -(*S*-L-cysteinyl)-(+)-catechin. These spectroscopic data are also identical with those reported by Torres et al. (17).

Compound **3** gave a blue coloration with the FeCl₃ reagent, suggesting the presence of a pyrogallol ring in the molecule (22). The HR-FAB-MS of **3** showed an [M + H]⁺ peak, which was 16 mass units more than that of **1**. The ¹H NMR spectrum of **3** (**Table 1**) was similar to that of **1**, except for the two proton singlet signals at δ 6.57 due to the 2- and 6-positions on the B-ring, thus leading to the conclusion that the structure was 4 β -(*S*-L-cysteinyl)-(-)-epigallocatechin (**3**).

Compound **4** showed an [M - H]⁻ peak corresponding to C₃₃H₃₀O₁₄NS in the negative HR-ESI-MS, suggesting a dimeric proanthocyanidin structure with a cysteine moiety. The ¹H NMR data for **4** (**Table 1**) showed a close analogy to those of procyanidin B-2, displaying broad singlets at δ 4.96 and 5.20 due to H-2 and -2', respectively, suggesting the presence of two epicatechin (2,3-*cis*) units. This was supported by ¹³C NMR chemical shifts of the C-2 signals (δ 74.6 and 76.0) (20). Because the ¹³C NMR chemical shifts attributable to the flavan

C rings were in good agreement with those observed in procyanidin B-2, the position and the configuration of the interflavanoid linkages were deduced to be C(4 β)-C(8) (23). In the ¹H NMR spectrum, the signal at δ 3.83 (1H, d, $J \approx 0$ Hz), assignable to flavan H-4 of the lower unit, indicated the presence of 4-substituted flavan rings with 2,3-*cis* and 3,4-*trans* configurations in the C-ring (24). The signals at δ 2.95, 3.44 (each 1H, br m, cys-H-3), and 4.14 (1H, dd, $J = 9, 4$ Hz, cys-H-2) in the ¹H NMR spectrum of **4** were assigned to the cysteine moiety. Accordingly, the structure of **4** was characterized as 4 β -(*S*-L-cysteinyloxy)-epicatechin-(4 β →8)-epicatechin.

Compound **5** showed an [M - H]⁻ peak corresponding to C₄₀H₃₄O₁₈NS by the negative HR-ESI-MS. The ¹H NMR spectrum of **5**, although all of the signals appeared to be broadened, showed a signal at δ 6.8 (2H) due to the gallic acid moiety. Treatment of **5** with tannase yielded gallic acid and a hydrolysate (**4**), which was identified on the basis of HR-ESI-MS (m/z 696.1407 [M - H]⁻, ¹H NMR (CD₃OD), and reverse-phase HPLC. The downfield shift of the H-3 signal in the ¹H NMR spectrum of **5** (δ 5.4) indicated the presence of the galloyl group at C-3 of the upper unit in **5**. On the basis of these findings, the structure of **5** was concluded as being 4 β -(*S*-L-cysteinyloxy)-epicatechin-(4 β →8)-epicatechin gallate (**5**).

Compound **6** showed an [M - H]⁻ peak for C₄₈H₄₂O₂₀NS by the negative HR-ESI-MS, which suggested that it was a trimeric proanthocyanidin structure with a cysteine moiety. In the ¹³C NMR spectrum of **6** (Table 2), chemical shifts [δ 75.2 and 76.4 (2C)] of C-2 suggested that compound **6** consists entirely of epicatechin units (20). The other signals attributable to the flavan C rings showed a close analogy to those of **4**, suggesting the position and the configuration of the interflavanoid linkage of three epicatechin units to be C(4 β)-C(8). The signal (δ 42.3) assignable to flavan C-4 of the terminal unit was in good agreement with that (δ 41.7) of **1**, suggesting that the cysteine moiety is located at C-4 of the terminal unit. On the basis of these findings, the structure of **6** was concluded as being 4 β -(*S*-L-cysteinyloxy)-epicatechin-(4 β →8)-epicatechin (Figure 1).

Assay of Each Component in Cys-OLG by Normal-Phase HPLC. As shown in Figure 2A, the peaks due to (+)-catechin and (-)-epicatechin (overlapped in peak A), (-)-epigallocatechin, and procyanidin B-2 were detected in GSP, and their contents were calculated as 3.5, 1.6, and 1.0%, respectively (Table 3). On the other hand, in cys-OLG, in addition to the above flavanols, the extra peaks corresponding to procyanidin B-1 and compounds **1-6** were newly observed, although compounds **1-3**, **5**, and **6** were individually observed as inseparable peaks (Figure 2B). The content of each component was calculated as shown in Table 3. It is thought that procyanidin B-1, which was not detected in GSP, was generated by cleavage of interflavanoid linkages, followed by a nucleophilic attack of the C-8 position of (+)-catechin on the C-4 position of procyanidin B-2 or (-)-epicatechin oligomers. The total contents of monomers and oligomers of cys-OLG (32.2%) were approximately 5 times greater than those of GSP (6.1%). As no monomers or dimers were detected in fraction I, it is suggested that the majority of fraction I is composed of proanthocyanidin polymers. Similarly, the main components of fractions II and III are monomers and oligomers, respectively (Table 3).

In Vitro and in Vivo Experiments. The in vitro antioxidant capacity of cys-OLG was compared with that of GSP in the TEAC assay. Antioxidant indices of 0.74 \pm 0.05 mM for GSP and 0.90 \pm 0.08 mM for cys-OLG were obtained, suggesting

Table 2. ¹³C Chemical Shifts Assignments for **1**, **3**, **4**, and **6**

C no.	1 ^a	3 ^b	4 ^a	6 ^c
C-2	74.7	75.7	76.0	76.4
C-3	70.6	71.9	72.0	71.7
C-4	41.7	42.9	36.1	36.7 ^d
C-5	156.1	157.2	156.6 ^d	155.8 ^e
C-6	94.5	96.2	95.3	95.7 ^f
C-7	157.1	158.2	156.5	157.1 ^g
C-8	96.4	99.0	95.3	97.0
C-9	157.7	159.1	156.5	155.9
C-10	99.0	99.6	99.0	99.7
B-C-1	130.8	131.1	130.7	131.1 ^h
B-C-2	115.8 ^d	107.1	114.7 ^e	114.8 ^j
B-C-3	144.6 ^e	146.7	144.6 ^f	144.9 ^j
B-C-4	144.7 ^e	133.6	144.2 ^f	144.7 ^k
B-C-5	115.9 ^d	146.7	115.9 ^e	115.8 ^m
B-C-6	119.1	107.1	119.2	118.8
C-2'			74.6	75.2
C-3'			69.9	72.8
C-4'			42.0	36.6 ^d
C-5'			156.5 ^d	155.8
C-6'			95.3	96.3 ^f
C-7'			156.6	157.0 ^g
C-8'			99.0	99.7
C-9'			156.6 ^d	155.9
C-10'			99.0	99.7
B'-C-1			130.7	131.6 ^h
B'-C-2			115.7	114.8
B'-C-3			144.6	145.0 ^j
B'-C-4			144.2	144.9 ^k
B'-C-5			115.7	115.7 ^m
B'-C-6			119.2	118.8
C-2''				76.4
C-3''				70.5
C-4''				42.3
C-5''				155.9 ^e
C-6''				96.3
C-7''				156.4
C-8''				99.7
C-9''				153.7
C-10''				95.7
B''-C-1				132.0 ^h
B''-C-2				115.0 ^j
B''-C-3				145.2 ^j
B''-C-4				144.7 ^k
B''-C-5				115.7
B''-C-6				118.8
cys-1	172.1	172.9	172.2	171.8
cys-2	53.7	54.7	53.7	54.0
cys-3	32.9	34.0	32.8	33.3

^a Measured in acetone-*d*₆-D₂O at 25 °C. ^b Measured in CD₃OD at 25 °C.

^c Measured in dimethyl sulfoxide-*d*₆-D₂O at 40 °C. ^{d-m} Values within the column are interchangeable.

that cys-OLG has a significantly higher antioxidant potential than GSP. In the same manner, the in vitro antioxidant capacities of the three fractions derived from cys-OLG were compared in the TEAC assay. Antioxidant indices of fraction I (0.78 \pm 0.04 mM), fraction II (0.99 \pm 0.09 mM), and fraction III (0.97 \pm 0.07 mM) were obtained, suggesting that fractions II and III, which were rich in monomers and oligomers, respectively, exhibited significantly higher antioxidant capacity than fraction I, which was rich in polymers.

For the in vivo experiment, GSP and cys-OLG were orally administered to rats at a dose of 10 mg/kg of body weight for a week (expt 1). The polyphenol concentrations in the GSP and cys-OLG groups were higher than that in the control group with a statistically significant difference. The antioxidant capacities (TEAC and LPO) for the GSP and cys-OLG groups were superior to those of the control group, but there was no significant difference between the control and the GSP groups. When the GSP and cys-OLG groups were compared, there was

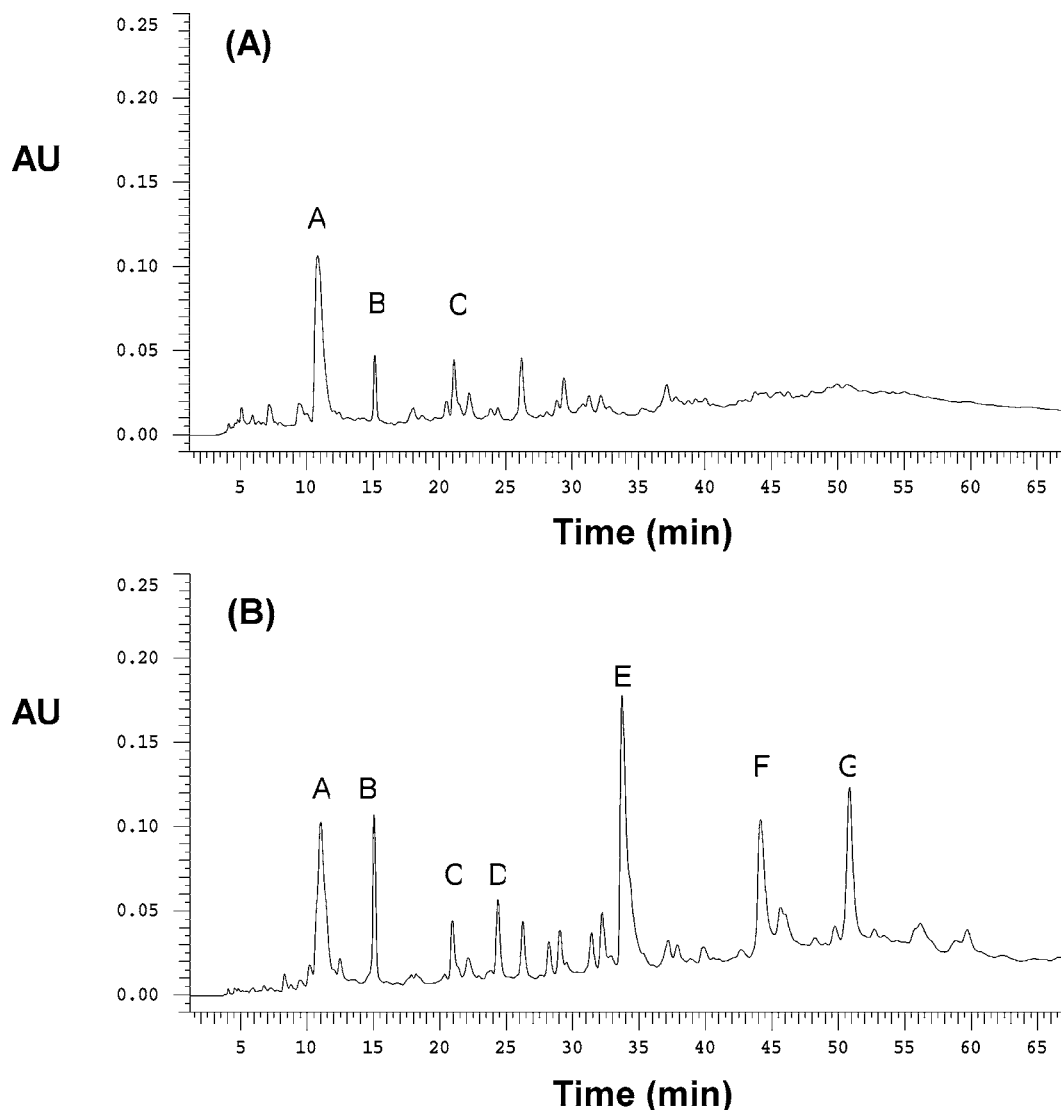


Figure 2. Normal-phase HPLC of GSP (A) and cys-OLG (B). Peak identification: A, (+)-catechin and (–)-epicatechin; B, (–)-epigallocatechin; C, procyanidin B-2; D, procyanidin B-1; E, 1 + 2 + 3; F, 4; G, 5 + 6.

Table 3. Contents (Percent) of Compounds in Each Fraction

	GSP	cys- OLG	fraction I	fraction II	fraction III
monomers					
(+)-catechin + (–)-epicatechin	3.5	3.8	ND ^a	7.6	ND
(–)-epigallocatechin	1.6	5.0	ND	8.3	ND
1 + 2 + 3		8.8	ND	65.8	ND
oligomers					
procyanidin B-1		1.8	ND	1.2	6.7
procyanidin B-2	1.0	1.1	ND	0.5	3.3
4		7.9	ND	ND	18.0
5 + 6		3.8	ND	ND	22.0
total	6.1	32.2	0	83.4	50.0

^a Not detected.

no significant difference in the polyphenol concentration and LPO, but for the TEAC assay, the cys-OLG group was superior to the GSP group, and the difference was statistically significant (Table 4). In the in vivo assessment of the polyphenol concentration and antioxidant capacities of the three fractions from cys-OLG (experiment 2), the monomer-rich fraction (II) and the oligomer-rich fraction (III) were more potent than the control and polymer-rich fraction (I) with statistically significant differences. The polymer-rich fraction (I) showed higher anti-

Table 4. Comparison of Polyphenol Concentration and Antioxidant Capacities (TEAC and LPO) in GSP and Cys-OLG Groups (Experiment 1) and Three Fractions from Cys-OLG (Experiment 2)^a

experiment	group	polyphenol ($\mu\text{g/mL}$)	TEAC (mM)	LPO (nmol/mL)
1	control	68.9 \pm 10.1	1.00 \pm 0.15	8.80 \pm 0.52
	GSP	99.1 \pm 18.2*	1.04 \pm 0.12	8.64 \pm 1.10
	cys-OLG	112.8 \pm 8.5*	1.21 \pm 0.05* [#]	7.91 \pm 0.83*
2	control	79.3 \pm 11.6	1.06 \pm 0.06	9.27 \pm 0.83
	fraction I	100.6 \pm 22.7	1.11 \pm 0.03	8.61 \pm 0.81
	fraction II	142.8 \pm 15.1 [†]	1.18 \pm 0.07 [†]	7.80 \pm 0.41 [†]
	fraction III	155.0 \pm 21.8 [†]	1.20 \pm 0.02 [†]	7.23 \pm 0.27 [†]

^a *, $p < 0.05$ versus control (experiment 1); #, $p < 0.05$ versus GSP (experiment 1); [†], $p < 0.05$ versus control, fraction I (experiment 2).

oxidant capacity than the control, but not significant. Although there was no significant difference between the monomer-rich fraction (II) and the oligomer-rich fraction (III) in the in vivo assessment of polyphenol contents, TEAC, and LPO, the tendency was for the oligomer-rich fraction (III) to be more potent than the monomer-rich fraction (II).

These results suggested that monomer and oligomer showed higher antioxidant capacity compared to the polymer in both in

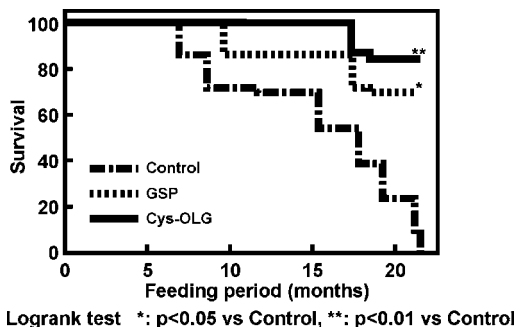


Figure 3. Survival study on normal mice.

vitro and in vivo studies, indicating that the major active components in cys-OLG are monomers and oligomers. The oligomer was the most potent in vivo, suggesting the procyanidin oligomers might be superior to polymers in bioavailability. Therefore, it is expected that cys-OLG might have various advantages due to a higher concentration of monomers and oligomers than conventional polyphenol products containing high concentrations of polymers.

Aging is an inevitable biological process for most organisms. One of the most accepted theories of aging emphasizes the oxidative damage induced by reactive oxygen species (ROS) that cause molecular and cellular damage (25, 26), which are important factors in determining the life span of living cells and the whole body. Therefore, it is suggested that preventing oxidative damage through the enhancement of antioxidant defenses may counteract aging and age-related disorders. On the basis of this information, a survival study using cys-OLG in normal mice was conducted. Control ddY mice started to die of natural causes at 7 months of age, whereas the age at which ddY mice of the GSP-treated and cys-OLG-treated groups started to die was 10 and 18 months, respectively (Figure 3). The control ddY mice survived to the age of 22 months, and the mean life span was 15 months. When all of the control ddY mice had died, the survival rates of the GSP-treated group and cys-OLG-treated group were 69 and 85%, respectively. The life span of ddY mice that were given GSP and cys-OLG was significantly prolonged (log-rank test; $p < 0.05$ in GSP, $p < 0.01$ in cys-OLG). Serum LPO levels after 6 months in the GSP and cys-OLG groups were lower than that of the control group with a statistically significant difference (data not shown). It is suggested that cys-OLG may have beneficial effects in preventing oxidative stress and that the reduction of oxidative stress as one of various factors to prolong the mean life span may contribute to prolongation of it.

In conclusion, the oligomerization process enabled the production of oligomeric proanthocyanidin-L-cysteine complexes by thiolysis using L-cysteine. Some of the proanthocyanidin-L-cysteine complexes, along with monomeric derivatives, were isolated, and their structures were confirmed on the basis of spectroscopic data and enzymatic hydrolysis. Cys-OLG contains higher amounts of monomers and oligomers than conventional polyphenol products containing high amounts of polymers such as GSP. The oligomeric proanthocyanidin-L-cysteine complexes showed higher bioavailability and antioxidant capacity and enhanced survival time in the animal test groups. In addition, it is suggested that the oligomeric complexes may help to prevent oxidative stress and may reduce free radical production. Cys-OLG may be superior to GSP in terms of these effects. As long as cys-OLG has no toxicity on living organisms, cys-OLG that was found to be superior to GSP in

terms of these effects could be a possible future candidate in food systems, pharmaceuticals, and cosmetic applications.

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