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α-Glucosidase Inhibiton and Antiglycation Activity of Laccase-Catalyzed Catechin Polymers

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ABSTRACT: Catechin polymers were produced by laccase (12 U/mL) in a mixture of sodium acetate buffer (1% (+)-catechin, 100 mM, pH 5) and methanol (buffer:methanol = 95:5, v/v). The freeze-dried catechin polymers were recovered from the precipitate after dialysis followed by centrifugation. Catechin polymers extracted with 20% ethanol had potent inhibitory activity against α-glucosidase with an IC_{50} value of 4 μg/mL, and they were present as a mixture of dimers, trimers, and tetramers. The antihyperglycemic effect of the catechin polymers was confirmed by an oral maltose tolerance test. The catechin polymers also had significantly improved antiglycation and superoxide dismutase-like activities compared to those of (+)-catechin. Since formation of advanced glycation end products and oxidative stress are accelerated in hyperglycemic conditions, we suggest that enzymatic production of catechin polymers could have a potential protective effect in type 2 diabetes, diabetic complications, and other free radical related diseases.

KEYWORDS: *catechin polymers, laccase, α-glucosidase inhibition, antihyperglycemic effect, antiglycation, SOD-like activity*

INTRODUCTION

Catechins are major polyphenols in green tea, and their beneficial health effects are well demonstrated.[1,2] Procyanidins, the naturally occurring catechin polymers, are formed by condensation of catechins with C4−C8 and/or C4−C6 bonds.[3] Procyanidins can regulate cell signaling either by direct interaction with signaling proteins or via cell membrane proteins and also exhibit strong protective effects against oxidation and adipocyte-related abnormalities.[4,5] This suggests that the physiological effects of catechin polymers vary depending on the structure and chain length of the catechin polymers.

Laccase (*p*-diphenol oxidase, EC 1.10.3.2) is a kind of polyphenol oxidase that can oxidize diverse phenolic substrates such as polyphenols, methoxy-substituted phenols, and diamines.[6] During oxidation, phenolic substrates are converted to free radicals, which undergo coupling-based polymerization.[7] Laccase-catalyzed catechin polymers had improved superoxide radical scavenging activity and xanthine oxidase inhibitory activity compared with those of catechin monomers.[8] Epicatechin oligomers prepared by horseradish peroxidase (HRP) had enhanced antiproliferative activity against human breast and colon cancer cells.[9,10]

Recently, it has been reported that polymerization of epicatechin preferentially occurred at the dihdroxybenzene of the A ring, and the lowest polar fraction of epicatechin oligomers resulted in the greatest ORAC antiradical activity compared to other fractions.[11] However, most enzymatic polymerization studies to date have been conducted from a chemistry or engineering perspective,[12,13] and the physiological activities of flavonoid polymers other than antioxidant activity have not been thoroughly explored.

Diabetes mellitus is a common endocrine disorder characterized by high blood sugar, and the global prevalence of type 2 diabetes is continuously rising. The rapid absorption of glucose is regarded as a risk factor for type 2 diabetes, and modulation of glucose absorption is therefore a potential strategy for the control of hyperglycemia. α-Glucosidase (EC 3.2.1.20) is an enzyme that catalyzes the exohydrolysis of 1,4-α-glucosidic linkages of carbohydrate and releases α-glucose for absorbance into the body. The inhibition of α-glucosidase

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activity can delay absorption of carbohydrates and reduce the postprandial elevation of blood glucose.[14] Under hyperglycemic conditions accumulation of advanced glycation end products (AGEs) can increase in the body and plays an important role in pathological diabetic complications, such as cataracts, retinopathy, and atherosclerosis.[15]

The objective of the present study was to evaluate the inhibitory effects of laccase-catalyzed catechin polymers on α-glucosidase and formation of AGEs. In addition, the molecular weight profile of the potent catechin polymer fraction was determined using LC−MS.

MATERIALS AND METHODS

Materials. Laccase (EC 1.10.3.2) from *Aspergillus oryzae* (Denilite IIS) was kindly provided by Novozymes (Bagasvaerd, Denmark). Black tea (Akbar Brothers Ltd., Colombo, Sri Lanka) was purchased from the local supermarket as a source of theaflavin. (+)-Catechin hydrate, α-glucosidase (EC 3.2.1.20), *p*-nitrophenyl α-D-glucopyranoside (*p*-NPG), nitroblue tetrazolium (NBT), nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), aminoguanidine (AG), dimethyl sulfoxide (DMSO), and all other chemicals were of analytical grade and were purchased from Sigma Chemical Co. (St. Louis, MO).

Synthesis and Fractionation of Catechin Polymers. Catechin polymers were synthesized by a previously described method with slight modification.[8] Catechin (1 g) was dissolved in a mixture of 0.1 M sodium acetate buffer (50 mL, pH 5) and methanol (95:5, v/v) with gentle stirring. Enzymatic polymerization was started by the addition of laccase (50 mL, 12 U/mL) to the reaction mixture and continued for 24 h at room temperature. The precipitate was recovered from the reaction mixture after centrifugation (2000*g* for 10 min; Hanil Science Industrial Co., Seoul, Korea) followed by two washings with methanol (5%, v/v). The precipitate was dialyzed (MWCO = 1000; Spectra/Por dialysis membrane, Spectrum Laboratories, Inc., CA) against distilled water and lyophilized. The freeze-dried catechin polymers were kept at −20 °C until further use. Catechin polymers (0.2 g) were extracted successively with ethanol (10%, 20%, 30%, 40%, 60%, and 100%, v/v; 50 mL) and acetone (80% and 100%, v/v; 50 mL). After two rounds of extraction with solvent under sonication (KODO Technical Research Co., Gyeonggi, Korea) for 30 min, the sample was centrifuged at 2000*g* for 10 min. The extraction was continued with the next solvent using the precipitate. Catechin polymers were recovered from the supernatant after every extraction.

Preparation of Theaflavins. Theaflavins were extracted from black tea by a previous method.[16] Briefly, black tea powder (100 g) was boiled in hot water (2.5 L) and filtered. Concentrated sulfuric acid (20 mL) was added to the filtrate, and the precipitate was recovered by centrifugation at 500*g*. The wet precipitate was extracted four times with ethyl acetate (150 mL). The combined ethyl acetate phases were washed with sodium bicarbonate solution (2.5%, w/v) and then sulfuric acid (0.1 M). The extract was redispersed in acetone (10 mL), and the precipitate was obtained after addition of chloroform (80 mL) followed by centrifugation. The theaflavin powder was obtained after removal of solvent using a rotary evaporator (Eyela, Tokyo, Japan). The total theaflavin content (the mixture of theaflavin, theaflavin gallate, and theaflavin digallate) was calculated using the molecular extinction coefficient reported previously.[16]

Characterization of Catechin Polymers. UV/vis spectra of the samples [monomeric (+)-catechin and catechin polymers] were obtained using a spectrophotometer (Ultrospec 2100 pro, Amersham Pharmacia Biotech, Little Chalfont, U.K.). The FT-IR spectra of the samples were taken using a Fourier transform infrared spectrometer (Vertex70, Bruker Optics, Ettlingen, Germany). The spectral data of each sample were collected in the range of 4000−400 cm^{-1} at room temperature. For background and sample readings 16 scans were performed at a nominal resolution of 4 cm^{-1}. Opus software (Bruker optics) was used for data acquisition and instrument control. The mass spectral analysis of catechin polymers (20% ethanol extract) was analyzed using a Waters 2695 separation module equipped with a photodiode array (PDA) detector and a Waters micromass ZQ mass spectrometer (Waters, Milford, MA). The sample (10 uL) was applied to a C-18 reversed-phase column (2.1 mm i.d. × 100 mm length, 3.5 μm particle size; Waters XTerra MS C18) and eluted with 40% methanol at a flow rate of 0.1 mL/min. PDA detection was monitored between 190 and 800 nm. The MS parameters are as follows: ionization mode, ES^-; capillary voltage, 3.0 kV; extractor voltage, 2 V; source temperature, 130 °C; desolvation temperature, 300 °C; desolvation N_2 flow, 300 L h^{-1}; cone N_2 flow, 50 L h^{-1}; scan range, 2−2000 amu; scan rate, 1 scan s^{-1}; cone voltage, 30 eV.

α-Glucosidase Inhibitory Activity. α-Glucosidase inhibitory activity was determined using a previously described method[17] with modifications. α-Glucosidase (0.6 U/mL) and substrate (*p*-nitrophenyl α-D-glucopyranoside, 0.07%) were dissolved in potassium phosphate buffer (100 mM, pH 6.8), and all samples were dissolved in DMSO. The reaction mixture containing the sample solution (50 μL), *p*-NPG solution (100 μL), and buffer (1 mL) was preincubated at 37 °C for 10 min, and α-glucosidase solution (50 μL) was added to the reaction mixture. After incubation at 37 °C for 15 min, the reaction was stopped by adding sodium hydroxide (1 N, 1 mL). The amount of *p*-nitrophenol released from *p*-NPG was determined using a spectrophotometer (Amersham Pharmacia Biotech) at 400 nm, and the α-glucosidase inhibitory activity was calculated as follows:

$$\alpha\text{-glucosidase inhibitory activity (\%)} = \left[\frac{\Delta\mathrm{Abs(control)} - \Delta\mathrm{Abs(sample)}}{\Delta\mathrm{Abs(control)}}\right] \times 100$$

By means of the inhibitory potency of the samples, the half-maximal inhibitory concentration (IC_{50}) was determined.

Antiglycation Assay. Antiglycation assays were performed using two model systems.[18] For the BSA−glucose assay, samples (100 μL) were added to a glycation reaction mixture (1 mL) containing BSA (50 mg/mL), fructose (0.2 M), and 0.02% (w/v) sodium azide in potassium phosphate buffer (100 mM, pH 7). In the BSA−MGO (methylglyoxal) assay, BSA (10 mg/mL) and MGO (25 mM) were used in the reaction mixture. After incubation of the reaction mixture at 37 °C for designated periods, the formation of total fluorescent AGEs was determined using a fluorescent microplate reader (Synergy HT, Biotek Instrument Inc., Winooski, VT) with an excitation wavelength of 360 and an emission wavelength of 460 nm. AG was used as a positive control. The inhibition activity was calculated using the following equation:

$$\text{inhibition (\%)} = \left[\frac{\Delta\mathrm{Abs(control)} - \Delta\mathrm{Abs(sample)}}{\Delta\mathrm{Abs(control)}}\right] \times 100$$

Superoxide Dismutase (SOD)-like Activity. SOD-like activity was determined by a previously described method with slight modification.[19] To prepare the reaction mixture, NBT (156 μM), NADH (468 μM), and PMS (60 μM) were dissolved in potassium phosphate buffer (100 mM, pH 7.4). The reaction mixture containing the sample (0.5 mL), NBT (0.5 mL), and NADH (0.5 mL) was preincubated at 37 °C for 5 min, and PMS (0.5 mL) was added to the reaction mixture. After incubation at 30 °C for 10 min, the absorbance at 560 nm was measured. SOD-like activity was calculated using the following equation:

$$\text{SOD-like activity (\%)} = \left[\frac{\Delta\mathrm{Abs(control)} - \Delta\mathrm{Abs(sample)}}{\Delta\mathrm{Abs(control)}}\right] \times 100$$

Animal Study. All animal procedures were approved by the Institutional Animal Care and Use Committees at Chonnam National University (Grant CNU IACUC-YB-2012-1). Six week old male C57BL/6 mice (25 ± 3 g) were purchased from Damul Science (Seoul, Korea) and maintained on a 12 h light−dark cycle at 24 °C. All mice were fed a standard chow diet (protein, 20%; carbohydrate, 64%;

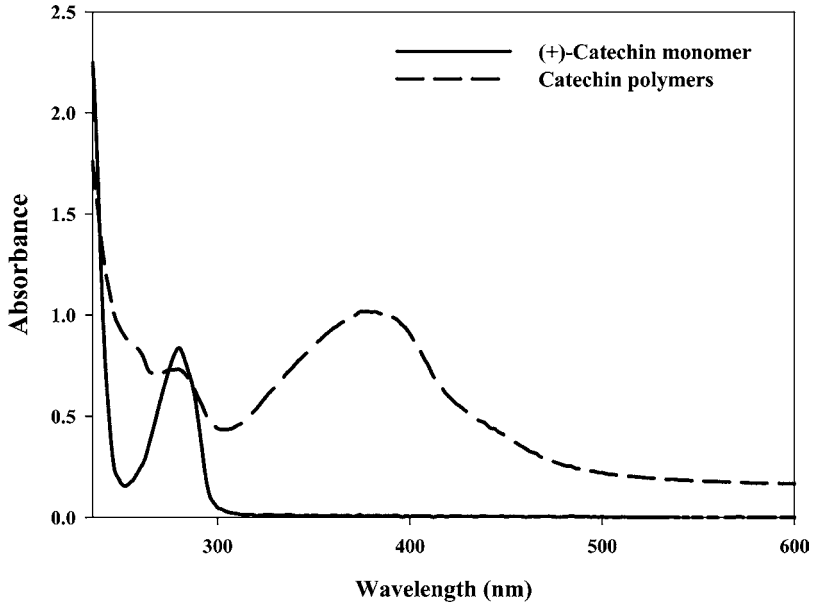

Figure 1. UV−vis spectra of (+)-catechin and catechin polymers synthesized by laccase. (+)-Catechin and catechin polymers (1%) were dissolved in methanol.

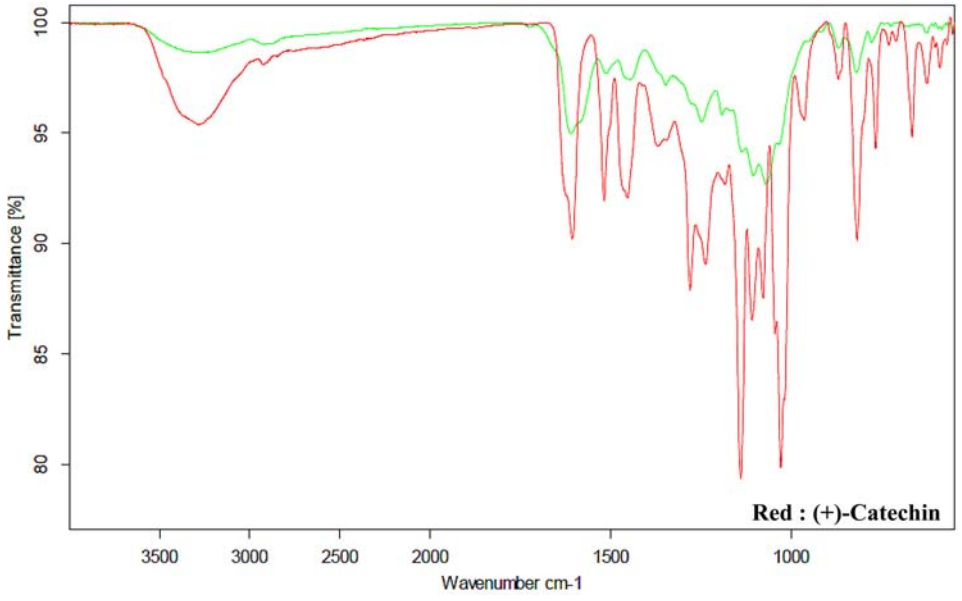

Figure 2. FT-IR spectra of (+)-catechin (red) and catechin polymers (green) synthesized by laccase. The spectral data of each sample (1% in methanol) were collected in the range of 4000−400 cm^{-1} at room temperature. For background and sample readings 16 scans were conducted.

fat, 16%; 4000 kcal/kg; Damul Science) for a week prior to the experiment. A single oral administration of sample (catechin monomer vs catechin polymers, 20 mg/kg) was achieved by oral gavage in mice that had been fasted for 16 h. After 5 min, maltose solution (1 g/kg of body mass) was given to the mice using oral gavage. Blood samples were collected by tail bleeding, and the blood glucose level was measured using an automatic One Touch glucometer (Lifescan, Milpitas, CA) at the indicated times.

Statistical Analysis. All experiments were performed at least in triplicate. Statistical analysis was performed by analysis of variance (ANOVA) via a statistical program package (SPSS, version 18.0, SPSS Inc., Chicago, IL). When ANOVA revealed a significant difference, Tukey's HSD test was used for multiple comparisons. The level of significant difference was set at $p < 0.05$.

RESULTS AND DISCUSSION

Synthesis of Catechin Polymers. The catechin polymers were synthesized by laccase at room temperature. The color of catechin turned dark brown as oxidation proceeded, and the yield of polymerization was typically greater than 60%. The development of a dark brown color indicates coupling and oxidation of *m*-diphenol to quinones. It has been reported that laccase is able to oxidize *o*- and *p*-diphenols.[20] The UV−vis spectra of (+)-catechin and catechin polymers synthesized by laccase are shown in Figure 1. The characteristic UV absorption peak of monomeric (+)-catechin at 280 nm disappeared, while a large broad absorption peak at 370 nm was observed by enzymatic polymerization. A similar transition of UV absorption spectra was reported in catechin polymers produced by enzymatic polymerization.[8,10,21]

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The FT-IR absorption spectra of (+)-catechin and catechin polymers are presented in Figure 2. The FT-IR spectrum of catechin polymers was broadened by enzymatic polymerization. Notably, the characteristic absorption regions for the OH group (3400−3100 cm^{-1}) became weak and stretched, indicating an involvement of molecular OH in hydrogen bond formation. Enzymatic oxidation also increased the region around 1600 cm^{-1} that corresponded to aromatic C═C vibration. This reflects an increase in aromatic ring structures in catechin polymers. The FTIR absorption spectrum of catechin polymers was similar to that of polymeric polyphenols in black tea.[22] On the basis of the above results, formation and recovery of catechin polymers were confirmed. Although the peak pattern and peak intensity of the FTIR spectra are informative, they only provide an overview of chemical bonds rather than their exact structure. We are currently using more highly purified catechin polymers to elucidate the precise chemical structures.

α-Glucosidase Inhibitory Effect of Catechin Polymers. The α-glucosidase inhibitory activity of catechin polymers was evaluated. (+)-Catechin barely inhibited α-glucosidase, whereas the α-glucosidase inhibitory activity of catechin polymers was remarkably increased by more than 70-fold (Table 1). To identify potent fractions, catechin polymers were successively extracted with different solvents, and the IC_{50} values of each fraction were compared. The 20% and 30% ethanol extracts were more effective than other fractions, and the 20% ethanol extract had the highest inhibitory activity, with an IC_{50} value of 4 μg/mL. The yield of the 20% ethanol extract was approximately 18% of the initial catechin polymers (data not shown).

Table 1. α-Glucosidase Inhibitory Activity (IC_{50}) of Catechin Polymers and Their Solvent Extracts

sample	IC_{50}[a] (μg/mL)	sample	IC_{50}[a] (μg/mL)
catechin monomer	>1200	40% ethanol extract	21
catechin polymers	17	60% ethanol extract	41
theaflavins[b]	54	100% ethanol extract	50
10% ethanol extract	18	80% acetone extract	185
20% ethanol extract	4	100% acetone extract	119
30% ethanol extract	11		

[a]Concentration required for 50% inhibition of the enzyme activity under the assay conditions. [b]Theaflavins were extracted from black tea and a mixture of theaflavin, theaflavin monogallate, and theaflavin digallate.

The molecular weight profile of the potent fraction was analyzed using LC−MS. As shown in Figure 3, the molecular weights of major catechin polymers obtained from 20% ethanol extraction were 575, 863, and 1151, and they were identified as dimers, trimers, and tetramers, respectively. These values were close to those reported for catechin trimers (m/z of 865) and tetramers (m/z of 1153) for the [M + H] species in FAB-MS.[23] This result suggests that catechin oligomers with 2−4 degrees of polymerization (DP) had greater potency against α-glucosidase than higher DP polymers. Although various structures of oligomeric (+)-catechin oxidation products can be generated by enzymatic oxidation, the repeated condensation structure between the A ring of one molecule and the B ring of another (head to tail polymerization) is a commonly found structure.[24,25]

Previously, α-glucosidase (maltase) inhibitory activities of catechin and theaflavins were compared.[26] According to the results, theaflavins had greater inhibitory activity than catechins. The authors maintained that a gallate moiety and free OH group at the 3′-position were required for potent maltase inhibition. It has been demonstrated that the α-glucosidase inhibitory activity of black or oolong tea was greater than that of green tea using an in vitro digestion study.[27] On the basis of the IC_{50} values, theaflavins prepared from black tea (54 μg/mL) had greater α-glucosidase inhibitory activity than catechin monomers, but theaflavins were less active than catechin polymers, especially those prepared with 20% ethanol extraction.

Flavan-3-ol refers to flavonoid molecules that have a characteristic saturated C-ring and 3-OH, and procyanidins indicate dimeric and oligomeric flavan 3-ols. Proanthocyanidin oligomers (DP = 3.3) isolated from perisimmon peel showed a stronger inhibitory activity than the polymers (DP > 3.3).[28] The potency of flavan-3-ols isolated from *Cynomorium songaricum* also increased as the oligomerization state of the catechins progressed from monomers to pentamers.[29]

The presence of functional groups at specific locations makes a significant impact on enzyme inhibition. Polyphenols have an ability to interact with an enzyme via hydrophobic interactions, hydrogen bonds, and π−π interactions of the aromatic ring.[30] The laccase-catalyzed catechin oligomers consisting of 2−4 monomer units possibly increase the hydrogen bond potential and therefore bind more effectively to α-glucosidase than the monomeric forms. In addition, the planarity of the molecule plays a crucial role in their potency against α-glucosidase. (+)-Catechin showed relatively weak inhibitory activity, with IC_{50} > 500 μM, while its planar analogues displayed much greater inhibitory activity, with IC_{50} values over a range between 0.7 and 47.5 μM.[31] Catechin with planar arrangements might facilitate access to the hydrophobic pockets in α-glucosidase.

Although many flavonoids show α-glucosidase inhibitory activity in vitro, only limited compounds maintain their activity in vivo. To validate their antidiabetic effects, the blood glucose level of mice fed with catechin polymers was monitored after a single oral administration of maltose (1 g/kg). As shown in Figure 4, mice fed on diets containing either catechin polymers or (+)-catechin had improved glucose tolerance compared with the control group. The glucose-lowering effects of catechin polymers at 30 and 90 min were significantly higher than that of monomeric (+)-catechin. This result suggests that catechin polymers effectively delay the rise in postprandial glucose in mice.

The α-glucosidase inhibiton of catechin polymers could explain their antihyperglycemic effect, but the exact mechanisms underlying the antihyperglycemic effect require further clarification. According to recent reports, reduced plasma glucose and glycosylated hemoglobin contents were found in diabetic rats by administration of green tea extract, catechin-based oligomeric procyanidins, or polymeric procyanidins.[32,33] This hypolglycemic effect was related to suppression of gluconeogenic enzymes such as glucose-6-phosphatase, fructose-1,6-bisphosphatase, and phosphoenol pyruvate carboxykinase. Also, the insulinotrophic effect of green tea extract was observed in the same studies. Consistent with our results, previous in vitro studies have reported that oolong tea extract, which contains catechin polymers, showed antihyperglycemic

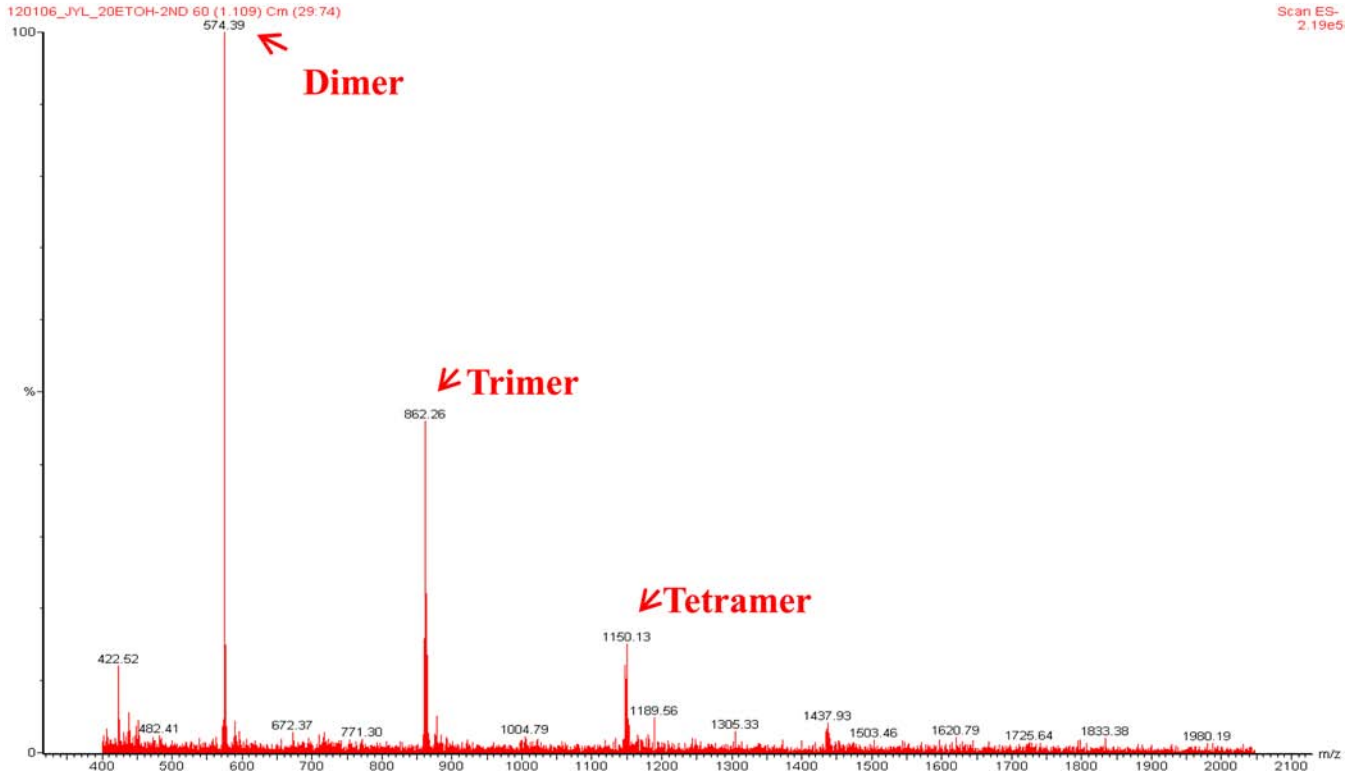

Figure 3. Molecular weight profile of catechin polymers in the 20% ethanol extract: dimer (m/z 575), trimer (m/z 863), tetramer (m/z 1151). The mass spectrum was obtained with negative mode ESI-MS.

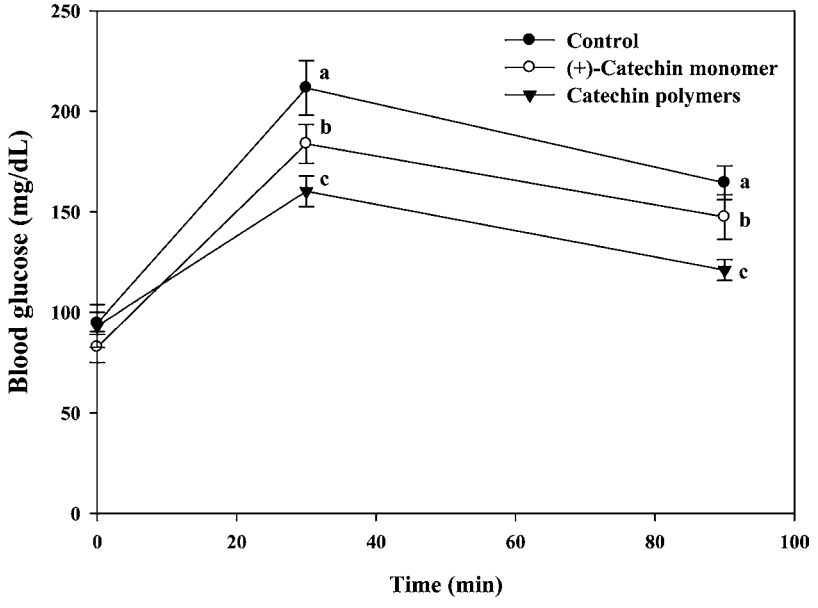

Figure 4. Effects of catechin polymers on the plasma glucose level after single oral administration of maltose in mice. A single oral administration of sample (catechin monomer vs catechin polymers, 200 mg/kg) was achieved by oral gavage in mice that had been fasted for 16 h. After 5 min, maltose solution (1 g/kg of body mass) was given to the mice using oral gavage. Each point represents the mean ± SD of five mice. Different letters at the data points indicate significant differences ($p < 0.05$).

effects in sucrose-, maltose-, or glucose-fed rats and in type 2 diabetes.[34,35]

Antiglycation Effect of Catechin Polymers. AGEs are the final heterogeneous products produced by a Maillard-type reaction between reducing sugar and proteins (or nucleic acid) containing an amino group. As AGEs are known to be involved in the development of the pathological process of various diseases, many efforts have focused on the isolation of AGE inhibitors from natural resources. In this regard, phenolic antioxidants are increasingly viewed as promising AGE inhibitors.[36] The antiglycation effect of catechin polymers has been examined in two model systems. In the BSA−fructose model, the AGE inhibitory activity increased in the order monomeric (+)-catechin, 20% ethanol extract of catechin

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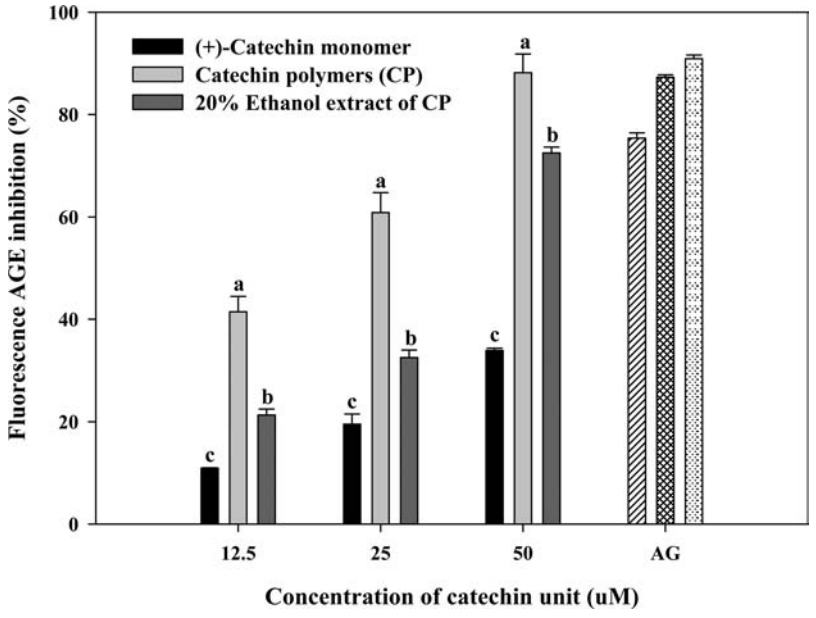

Figure 5. Inhibitory effect of catechin polymers on AGE formation in the BSA−fructose model at 37°C for 3 days. AG (left hatched bar, 1 mM; middle criss-crossed bar, 2.5 mM; right dotted bar, 5 mM) was used as a positive control. The results are expressed as means ± SD. Bars with different letters indicate significant differences ($p < 0.05$).

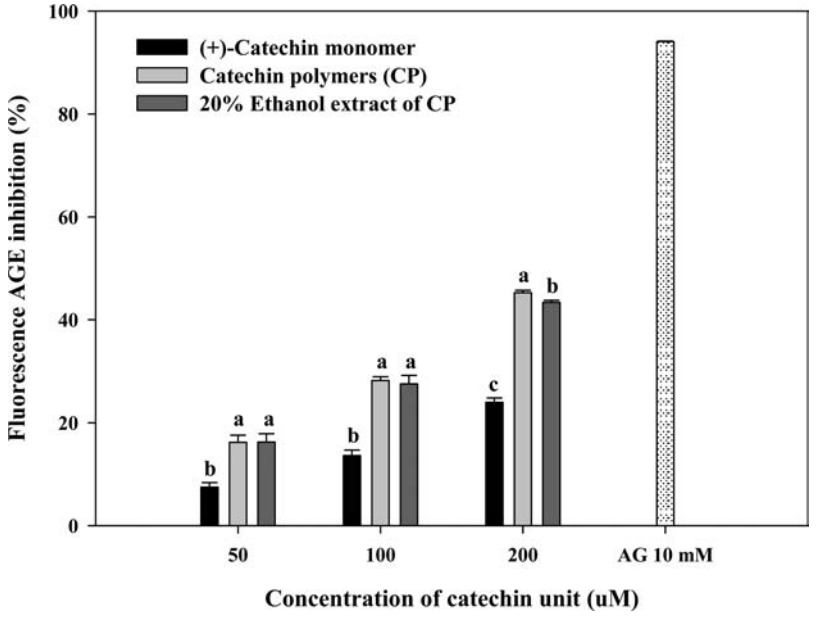

Figure 6. Inhibitory effect of catechin polymers on AGE formation in the BSA−methylglyoxal model at 37°C for 7 days. Aminoguanidine (10 mM) was used as a positive control. The results are expressed as means ± SD. Bars with different letters indicate significant differences ($p < 0.05$).

polymers, and whole catechin polymers (Figure 5). Considering that whole catechin polymers have a greater molecular weight that those isolated from the 20% ethanol extract, the antiglycation activity appears to increase proportionally with increased DP of the catechin polymer.

MGO is a major precursor of AGEs and is produced by various biochemical pathways. It was reported that the concentrations of glyoxal and MGO were elevated in diabetic patients and were associated with high oxidative stress.[37] In the BSA−MGO model, catechin polymers and the 20% ethanol extract were equally able to prevent formation of AGEs, while monomeric (+)-catechin showed significantly lower antiglycation activity (Figure 6).

The reactive carbonyl trapping ability of tea polyphenols was evaluated.[38] Theaflavins resulted in greater MGO reduction ability than monomeric catechins, such as epicatechin (EC), epicatechin-3-gallate (ECG), epigallocatechin (EGC), and epigallocatechin-3-gallate (EGCG). It has been reported that catechin, epicatechin, and procyanidin B2 (a dimer-type proanthocyanidin) isolated from cinnamon bark extract exhibited antiglycation activities.[39] All compounds displayed greater MGO trapping activity than AG, the well-known MGO scavenger, and procyanidin B2 showed the greatest inhibition of AGE formation and MGO trapping activity. The authors also commented that proanthocyanidin oligomers and polymers maintained antiglycation activity.

Although the exact mechanisms are not clear, the blocking of sugar attachment to proteins and scavenging of reactive dicarbonyls and free radicals are suggested as the principal mechanisms underlying the inhibiton of AGE formation.[36] The antiglycation activities of 25 plant ethanol extracts correlated highly with antioxidant activity determined with lipid peroxidation (r =0.75) or DPPH radical scavenging activity (r = 0.76).[40]

Superoxide radicals are naturally generated in the metabolism of the human body. The scavenging of superoxide radicals is physiologically important since these radical anions are potential precursors of highly reactive radical species, such as hydroxyl radicals.[41] The SOD-like activity of catechin polymers was also examined. As shown in Figure 7, catechin polymers

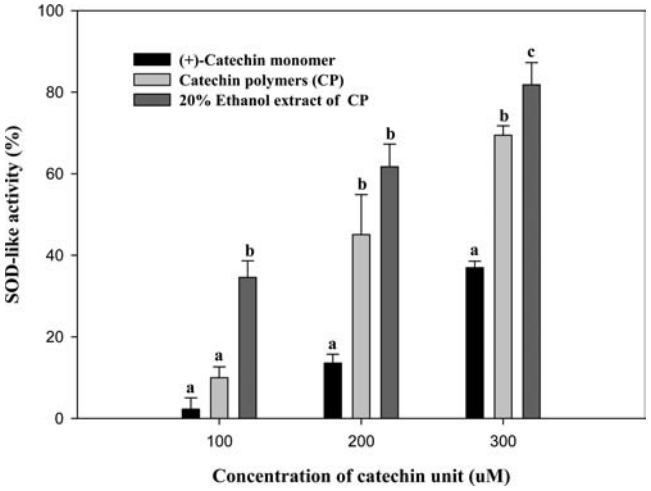

Figure 7. Superoxide dismutase-like activity of (+)-catechin and catechin polymers. The results are expressed as means ± SD. Bars with different letters indicate significant differences (p < 0.05).

resulted in significantly higher SOD-like activity than monomeric (+)-catechin, and 20% ethanol extract showed the greatest SOD-like activity. It was reported that the SOD-like activity of procyanidins increased proportionally with the degree of polymerization, at least up to the trimeric state.[42] However, procyanidin oligomers above the trimeric state had decreased SOD-like activity. This trend was very similar to tha tof catechin polymers synthesized by laccase. On the basis of the structure−activity relationship, catechol B rings of flavonoids are essential for strong radical scavenging and chain breaking activity.[43,44] The SOD-like activity of catechin polymers was improved about 4−5-fold by acetaldehyde condensation of catechin monomer.[45] The authors reported that the increase of repeating catechol B rings in catechin polymers was responsible for the amplification of superoxide radical scavenging activity. It has been demonstrated that the level of serum SOD in rats fed various tea leaves increased in the order green tea < oolong tea < black tea.[46] This result suggests that fermented tea leaves containing oxidized catechin polymers consistently have better radical scavenging activity than unfermented green tea in vivo.

The antioxidant activity of catechin polymers in the human body also varies depending on their bioavailability. Generally, compounds with low molecular weight seem to be absorbed more easily than those with high molecular weight in the digestive tract. Procyanidin dimers and trimers were absorbed across monolayers in human intestinal epithelial cells (Caco-2) without difficulty.[47] Procyanidins in the form of dimers, trimers, and tetramers were directly absorbed in the digestive tract and were not degraded into lower molecular weight compounds in an animal study.[48] On the basis of the above studies, catechin oligomers with low DP could easily be absorbed in the digestive tract and might act as potent antioxidants in the human body after consumption.

Laccase-catalyzed polymerization of (+)-catechin significantly improved antidiabetic potential. Catechin polymers, and particularly the 20% ethanol extract containing 2−4 monomer units, exerted potent α-glucosidase inhibitory activity, with an IC_{50} of 4 μg/mL. The antihyperglycemic effect of catechin polymers was confirmed by an oral glucose (maltose) tolerance test. The catechin polymers also had significantly improved antiglycation and SOD-like activities compared to those of (+)-catechin. Considering that AGE formation and oxidative stress are accelerated during hyperglycemia, enzyme-produced catechin polymers may have a potential protective effect in type 2 diabetes, diabetic complications, and other free radical related diseases.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

AGEs, advanced glycation end products; FT-IR, Fourier transform infrared; LC−MS, liquid chromatography−mass spectrometry; MGO, methylglyoxal; SOD, superoxide dismutase

REFERENCES

(1) Thielecke, F.; Boschmann, M. The potential role of green tea catechins in the prevention of the metabolic syndrome—a review. *Phytochemistry* **2009**, *70*, 11−24.

(2) Dufresne, C. J.; Farnworth, E. R. A review of latest findings on the health promotion properties of tea. *J. Nutr. Biochem.* **2001**, *12*, 404−421.

(3) Koga, T.; Moro, K.; Nakamori, K.; Yamakoshi, J.; Hosoyama, H.; Kataoka, S.; Ariga, T. Increase of antioxidative potential of rat plasma by oral administration of proanthocyanidin-rich extract from grape seeds. *J. Agric. Food Chem.* **1999**, *47*, 1892−1897.

(4) Fraga, C. F.; Oteiza, P. I. Dietary flavonoids: role of (−)-epicatechin and related procyanidins in cell signaling. *Free Radical Biol. Med.* **2011**, *51*, 813−823.

(5) Pinent, M.; Blade, C.; Salvado, M. J.; Blay, M.; Pujadas, G.; Fernandez-Larrea, J.; Arola, L.; Ardevol, A. Procyanidin effects on adipocyte-related pathologies. *Crit. Rev. Food Sci. Nutr.* **2006**, *46*, 543−550.

(6) Thurston, C. F. The structure and function of fungal laccases. *Microbiology* **1994**, *140*, 19−26.

(7) Yaropolov, A. I.; Skorobogatko, O. V.; Vartanov, S. S.; Varfolomeyev, S. D. Laccase-properties, catalytic mechanism, and applicability. *Appl. Biochem. Biotechnol.* **1994**, *49*, 257−280.

(8) Kurisawa, M.; Chung, J. E.; Uyama, H.; Kobayashi, S. Laccase-catalyzed synthesis and antioxidant property of poly(catechin). *Macromol. Biosci.* **2003**, *3*, 758−764.
(9) Nagarajan, S.; Nagarajan, R.; Braumhut, S. J.; Bruno, F.; McIntosh, D.; Samuelson, L.; Kumar, J. Biocatalytically oligomerized epicatechin with potent and specific anti-proliferative activity for human breast cancer cells. *Molecules* **2008**, *13*, 2704−2716.
(10) Bruno, F. F.; Nagarajan, S.; Nagarajan, R.; Kumar, J.; Samuelson, L. A. Biocatalytic synthesis of water-soluble oligo(catechins). *J. Macromol. Sci.* **2005**, *42*, 1547−1554.
(11) Racicot, K.; Favreau, N.; Fossey, S.; Grella, A. R.; Ndou, T.; Bruno, F. F. Antioxidant potency of highly purified polyepicatechin fractions. *Food Chem.* **2012**, *130*, 902−907.
(12) Hollmann, F.; Arends, I. W. C. E. Enzyme initiated radical polymerizations. *Polymers* **2012**, *4*, 759−793.
(13) Kobayashi, S.; Uyama, H.; Kimura, S. Enzymatic polymerization. *Chem. Rev.* **2001**, *101*, 3793−3813.
(14) Lebovitz, H. E. Alpha-glucosidase inhibitors. *Endocrinol. Metab. Clin. North Am.* **1997**, *26*, 539−551.
(15) Ahmed, N. Advanced glycation endproducts—role in pathology of diabetic complications. *Diabetes Res. Clin. Pract.* **2005**, *67*, 3−21.
(16) Clark, K. J.; Grant, P. G.; Sarr, A. B.; Belakere, J. R.; Swaggerty, C. L.; Phillips, T. D.; Woode, G. N. An in vitro study of theaflavins extracted from black tea to neutralize bovine rotavirus infections. *Vet. Microbiol.* **1998**, *63*, 147−157.
(17) Matsui, T.; Yoshimoto, C.; Osajima, K.; Oki, T.; Osajima, Y. In vitro survey of α-glucosidase inhibitory food components. *Biosci., Biotechnol., Biochem.* **1996**, *60*, 2019−2022.
(18) Wu, C.-H.; Yen, G.-C. Inhibitory effect of naturally occurring flavonoids on the formation of advanced glycation endproducts. *J. Agric. Food Chem.* **2005**, *53*, 3167−3173.
(19) Nishimiki, M.; Appaji Rao, N.; Yagi, K. The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. *Biochem. Biophys. Res. Commun.* **1972**, *46*, 849−853.
(20) Pourcel, L.; Routaboul, J.-M.; Cheynier, V.; Lepiniec, L.; Debeaujon, I. Flavonoid oxidation in plants: from biochemical properties to physiological functions. *Trends Plant Sci.* **2007**, *12*, 29−36.
(21) Mejias, L.; Reihmann, M. H.; Sepulveda-Boza, S.; Ritter, H. New polymers from natural phenols using horseradish or soybean peroxidase. *Macromol. Biosci.* **2002**, *2*, 24−32.
(22) Krishnan, R.; Maru, G. B. Isolation and analyses of polymeric polyphenol fractions from black tea. *Food Chem.* **2006**, *94*, 331−340.
(23) Hamada, S.; Kontani, M.; Hosono, H.; Ono, H.; Tanaka, T.; Ooshima, T.; Mitsunaga, T.; Abe, I. Peroxidase-catalyzed generation of catechin oligomers that inhibit glucosyltransferase from *Streptococcus sorbrinus*. *FEMS Microbiol. Lett.* **1996**, *143*, 35−40.
(24) Guyot, S.; Vercauteren, J.; Cheynier, V. Structural determination of colorless and yellow dimers resulting from (+)-catechin coupling catalyzed by gape polyphenoloxidase. *Phytochemistry* **1996**, *42*, 1279−1288.
(25) Tanaka, T.; Kouno, I. Oxidation of tea catechins: chemical structures and reaction mechanism. *Food Sci. Technol. Res.* **2003**, *9*, 128−133.
(26) Matsui, T.; Tanaka, T.; Tamura, S.; Toshima, A.; Tamaya, K.; Miyata, Y.; Tanaka, K.; Matsumoto, K. α-Glucosidase inhibitory profile of catechins and theaflavins. *J. Agric. Food Chem.* **2007**, *55*, 99−105.
(27) Koh, L. W.; Wong, L. L.; Loo, Y. Y.; Kasapis, S.; Huang, D. Evaluation of different teas against starch digestibility by mammalian glycosidases. *J. Agric. Food Chem.* **2010**, *58*, 148−154.
(28) Lee, Y. A.; Cho, E. J.; Tanaka, T.; Yokozawa, T. Inhibitory activities of proanthocyanidins from persimmon against oxidative stress and digestive enzymes related to diabetes. *J. Nutr. Sci. Vitaminol.* **2007**, *53*, 287−292.
(29) Ma, C. M.; Sato, N.; Li, X. Y.; Nakamura, N.; Hattori, M. Flavan-3-ol contents, anti-oxidative and α-glucosidase inhibitory activities of *Cynomorium songaricum*. *Food Chem.* **2010**, *118*, 116−119.
(30) Siebert, K. J.; Troukhanova, N. V.; Lynn, P. Y. Nature of polyphenols−protein interactions. *J. Agric. Food Chem.* **1996**, *44*, 80−85.
(31) Hakamata, W.; Nakanishi, I.; Masuda, Y.; Shimizu, T.; Higuchi, H.; Nakamura, Y.; Saito, S.; Urano, S.; Oku, T.; Ozawa, T.; Ikota, N.; Miyata, N.; Okuda, H.; Fukuhara, K. Planar catechin analogues with alkyl side chains: a potent antioxidant and an α-glucosidase inhibitor. *J. Am. Chem. Soc.* **2006**, *128*, 6524−6525.
(32) Sundaram, R.; Naresh, R.; Shanthi, P.; Sachdanandam, P. Modulatory effect of green tea extract on hepatic key enzymes of glucose metabolism in streptozotocin and high fat diet induced diabetic rats. *Phytomedicine* **2013**, *20*, 577−584.
(33) Huang, P.-L.; Chi, C.-W.; Liu, T.-S. Areca nut procyanidins ameliorate streptozocin-induced hyperglycemia by regulating gluconeogenesis. *Food Chem. Toxicol.* **2013**, *55*, 137−143.
(34) Nakahara, K.; Izumi, R.; Kodama, T.; Kiso, Y.; Tanaka, T. Inhibition of postprandial hyperglycaemia by oolong tea extract (OTE). *Phytother. Res.* **2006**, *8*, 433−435.
(35) Hosoda, K.; Iha, M.; Wang, M.-F.; Clevidence, B.; Liao, M.-L.; Yamamota, S.; Chuang, C.-K. Antihyperglycemic effect of oolong tea in type 2 diabetes. *Diabetes Care* **2003**, *26*, 1714−1718.
(36) Peng, X.; Ma, J.; Chen, F.; Wang, M. Naturally occurring inhibitors against the formation of advanced glycation end-products. *Food Funct.* **2011**, *2*, 289−301.
(37) Lapolla, A.; Flamini, R.; Vedova, A. D.; Senesi, A.; Reitano, R.; Fedele, D.; Basso, E.; Seraglia, R.; Traldi, P. Glyoxal and methylglyoxal levels in diabetic patients: quantitative determination by a new GC/MS method. *Clin. Chem. Lab. Med.* **2005**, *41*, 1166−1173.
(38) Lo, C.-Y.; Li, S.; Tan, D.; Pan, M.-H.; Sang, S.; Ho, C.-T. Trapping reactions of reactive species with tea polyphenols in simulated physiological conditions. *Mol. Nutr. Food Res.* **2006**, *50*, 1118−1128.
(39) Peng, X.; Cheng, K.-W.; Ma, J.; Chen, B.; Ho, C.-T.; Lo, C.; Chen, F.; Wang, M. Cinnamon bark proanthocyanidins as reactive carbonyl scavengers to prevent the formation of advanced glycation endproducts. *J. Agric. Food Chem.* **2008**, *56*, 1907−1911.
(40) Kim H, Y.; Lim, K. Protein glycation inhibitory and antioxidative activities of some plant extracts in vitro. *J. Agric. Food Chem.* **2003**, *51*, 1586−1591.
(41) Kanatt, S. R.; Chander, R.; Sharma, A. Antioxidant potential of mint (*Mentha spicata* L.) in radiation-processsed lamb meat. *Food Chem.* **2007**, *100*, 451−458.
(42) Lu, Y.; Foo, L. Y. Antioxidant radical scavenging activities of polyphenols from apple pomace. *Food Chem.* **2000**, *68*, 81−85.
(43) Salah, N.; Miller, N. J.; Paganga, G.; Tijburg, L.; Bolwell, G. P.; Rice-Evans, C. Polyphenolic flavanols as scavengers of aqueous phase radicals and as chain breaking antioxidants. *Arch. Biochem. Biophys.* **1995**, *322*, 339−346.
(44) Cos, P.; Ying, L.; Calomme, M.; Hu, J. P.; Cimanga, K.; Poel, B. V.; Pieters, L.; Vlietinck, A. J.; Berghe, D. V. Structure−activity relationship and classification of flavonoids as inhibitors of xanthine oxidase and superoxide scavengers. *J. Nat. Prod.* **1998**, *61*, 71−76.
(45) Chung, J. E.; Kurisawa, M.; Kim, Y.-J.; Uyama, H.; Kobayashi, S. Amplification of antioxidant activity of catechin by polycondensation with acetaldehyde. *Biomacromolecules* **2004**, *5*, 113−118.
(46) Kuo, K.-L.; Weng, M.-S.; Chiang, C.-T.; Tsai, Y.-J.; Lin-Shiau, S.-Y.; Lin, J.-K. Comparative studies on the hypolipidemic and growth suppressive effects of oolong, black, pu-erh, and green tea leaves in rats. *J. Agric. Food Chem.* **2005**, *53*, 480−489.
(47) Deprez, S.; Mila, I.; Huneau, J.; Tome, D.; Scalbert, A. Transport of proanthocyanidin dimer, trimer, and polymer across monolayers of human intestinal epithelial Caco-2 cells. *Antioxid. Redox Signaling* **2001**, *3*, 957−967.
(48) Shoji, T.; Masumoto, S.; Moriichi, N.; Akiyama, H.; Kanda, T.; Ohtake, Y.; Goda, Y. Apple procyanidin oligomers absorption in rats after oral administration: analysis of procyanidins in plasma using the porter method and high-performance liquid chromatography/tandem mass spectrometry. *J. Agric. Food Chem.* **2006**, *54*, 884−892.