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Antioxidant Activity of Theaflavin and Thearubigin Separated from Korean Microbially Fermented Tea

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Abstract

Theaflavins (TF) and thearubigins (TR) were separated from Korean microbially fermented tea leaves. Contents of TF (74.4 μM/g) and TR (37.2%) were higher than reported for black tea fermented by oxidase. Antioxidant activities of TF, TR and EGCG were analyzed and protective effects of COS-7 cells against copper and cadmium-induced toxicity were investigated. TF and TR exhibited good inhibition rates of about 85~90% for antioxidant and scavenging activities of free radicals and protected COS-7 cells against apoptosis or damage caused by stress, such as cadmium and copper-oxidative injury, free radicals etc. These results indicate that TF, TR and EGCG have antioxidant and scavenging activities against free radicals and protect COS-7 cells from Cu, Cd induced injury.

Key words: theaflavins (TF), thearubigins (TR), COS-7 cell, antioxidant activity, microbially fermented tea

INTRODUCTION

Tea leaves are rich in catechins, during fermentation of tea leaves catechins come in contact with polyphenol oxidase and microorganisms resulting in the formation of theaflavins (TF) and thearubigins (TR). During fermented tea manufacture, most of the catechin mass is converted to a well defined group of polyphenolic compounds know as TF and TR. Fermented tea contains major amounts of TR and TF, which are known to possess antimutagenic and antineoplastic activities (1). Korean fermented tea manufacture is carried out by a series of processes on fresh tea leaves, involving withering, rolling, fermentation and drying. All manufacturing processes are by hand. Fermentation is one of the critical steps. After microorganisms are inoculated, tea is fermented for 6 month. EGCG is the polyphenol component of green tea and was reported to inhibit LDL oxidation in vitro by scavenging oxygen radicals and chelating metal ions (2). Recently, it is increasingly being acknowledged that natural active components and biological activities of green tea have beneficial health effects.

Metal-induced lipid per-oxidation of the mitochondrial membrane may cause an uncoupling of oxidative phosphorylation, disruption of electron transport and ultimately result in cytotoxicity (3). Copper is a redox-active metal capable of catalyzing the formation of hydroxyl radicals; cadmium may induce oxidative stress by depleting intracellular antioxidants (4). Soluble cadmium salts accumulate and result in toxicity to liver, kidneys, brain and lungs, but does not appear to generate free radicals (5). Furthermore, various studies (6,7) have suggested that the ability to generate reactive oxygen species by redox cycling quinines and related compounds may require metal ions such as cadmium and copper. Studies by Fariss (8) have shown that free radical scavengers and antioxidants are useful in protecting against cadmium toxicity. Antioxidants reduce the cytotoxicity of hydrogen peroxide in endothelial cells and interrupt the propagation of lipid per-oxidation in the plasma membrane (9). This paper reports the formation of TF and TR during fermentation with microorganisms, and their protective effects of COS-7 cells and antioxidant activities.

MATERIALS AND METHODS

Manufacture of microbial fermented tea

Korean fermented tea is a microbial fermented tea which is inoculated with a wheat bran starter containing the fungus, *Aspergillus wentii* KOFRI 0341, which has potent α-amylase (5.71 U/g), pectinase (910.56 U/g) and polyphenol oxidase (PPO, 4.14 U/g solid) enzymes, isolated from Korean natural fermented tea. Green tea leaves were plucked from the Suncheon city (Jeonnam in Korea) wild tea-producing areas in June, 2005. Fresh tea leaves were withered and then subjected to the rolling

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process. Rolled leaves were inoculated with micro-organisms and fermented for 6 months. Fermented teas were dried in an incubator, used for analysis and studied for biological activity.

Analysis of total TF contents

Total TF were determined by the Flavognost method (10). The percentage of dry matter in the sample was determined by oven-drying. A tea infusion was made with 375 mL of boiling water, added from an overhead boiler into a tare flask, and 9 g of tea. The flask was shaken for 10 min, the infusion filtered rough cotton wool, and allowed to cool to room temperature; 10 mL was then pipetted into 10 mL of isobutyl methyl ketone (4-methylpentan-2-one, IBMK: ACS standard from Merck). The mixture was shaken for 10 min and allowed to stand until the layers separated. Two milliliters of the upper layer was pipetted into a test tube, followed by 4 mL ethanol and 2 mL Flavognost reagent (2 g diphenylboric acid-2-aminoethyl ester dissolved in 100 mL ethanol). The contents were mixed and color allowed to develop for 15 min. The absorbance (A) at 625 nm was read against an IBMK/ethanol (1:1, v/v) blank.

Spectrophotometer measurements of total TR

The method of Roberts and Smith (11) was used to determine total TR. Fifty milliliters of the cool, well-shaken and filtered standard tea infusion from TF analysis were mixed with 50 mL isobutylmethylketone (IBMK) and gently shaken to avoid formation of an emulsion. The layers were allowed to separate and 4 mL portion of the IBMK layer was taken and made to 25 mL with methanol in a volumetric flask (Solution A).

Twenty-five milliliters of the remaining initial IBMK layer were taken in a separate flask and mixed with 25 mL of 2.5% aqueous sodium hydrogen carbonate. The mixture was vigorously shaken before the layers were allowed to separate and the aqueous layer discarded. A 4 mL portion of the washed IBMK layer was made to 25 mL with methanol (Solution B).

Two milliliters of a saturated oxalic acid aqueous solution and 6 mL of water were added to a 2 mL portion of the aqueous layer left from the first extraction with IBMK, and diluted to 25 mL with methanol (Solution C).

The absorbencies A_A, A_B, A_C of solutions A, B and C at 380 nm were obtained using a CE 393 Cecil digital grating spectrophotometer with distilled water as the blank. Each fermented tea sample was extracted in triplicate for the determination of the TR fractions and levels.

By following the earlier procedures for solvent partitioning of fermentation tea liquor components and based on the known mean absorbance of the TR fractions at 380 nm of 0.733 (Roberts & Smith) (11), the following equation for estimating total TR was derived: At 380 nm % TR (Total) = $(375 \times 0.02 \times 6.25[2\ A_C + A_A - A_B])$ $/(0.733 \times 9 \times DM/100)$.

Cytotoxicity assays using COS-7 cells (monkey kidney fibroblast)

Cytotoxicity assays of COS-7 cells (monkey kidney fibroblast) were performed as described by Shokri (12). Cells were seeded with 1×10^6 cells and incubated for 24 hr. Prior to the addition of metal, various concentrations of samples were added to the culture medium. Following a 2-hr incubation, copper sulfate or cadmium chloride was added, and the cells grown for an additional 24 hr. Each treatment was performed in four replicates.

Hydroxyl-radicals scavenging activities

Hydroxyl radical scavenging activity was measured according to the method of Halliwell (13). One milliliter of the final reaction solution consisted of aliquots (0.5 mL) of various concentrations of the sample, 1 mM $FeCl_3$, 1 mM EDTA, 20 mM H_2O_2, 1 mM ascorbic acid, and 30 mM deoxyribose in potassium phosphate buffer. The reaction mixture was incubated for 1 hr at 37°C, and further heated in a boiling water-bath for 15 min after addition of 1 mL of 2.8% (w/v) 2-thiobarbituric acid. The color development was measured at 532 nm against a blank containing phosphate buffer.

Antioxidant activities using the β-carotene linoleate model system

Antioxidant activity was evaluated using the β-carotene linoleate model system (14). A solution of β-carotene was prepared by dissolving 2 mg of β-carotene in 10 mL of chloroform. Two milliliter of this solution was pipetted into a 100 mL round bottom flask. After the chloroform was removed under vacuum, 40 mg of purified linoleic acid, 400 mg of tween 40 emulsifier, and 100 mL of aerated distilled water were added to the flask. Aliquots (4.8 mL) of this emulsion were transferred into different test tubes containing different concentration of the sample. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm using a spectrophotometer (CE2021, CECIL, England). The tubes were placed at 50°C in a water bath. Measurement of absorbance was continued until the color of β-carotene disappeared, a blank, devoid of β-carotene, was prepared for background subtraction.

Statistical analysis

Values are expressed as the mean ± SD. The significance of the difference from the respective controls for each experiment was determined by ANOVA. $P<0.05$ was regarded as a statistically significance.

Table 1. Contents of TF and TR from Korean microbially fermented tea

	TF (μmol/g)	TR (%)
Control	0	0
Fermented tea	74.41±4.8	37.19±3.5

The results are expressed as means±SD of triplicate assays.

RESULTS AND DISCUSSION

The TF and TR contents of the fermented tea samples are showed in Table 1. Contents of TF (74.4 μM/g) and TR (37.2%) in the Korean fermented tea were higher than those (TF: 28.3 μM/g, TR: 16.9%) of black tea fermented by oxidase, as previously reported by Martin et al. (15). Microbial fermentation and the long fermentation time led to higher TF and total TR contents. Protective effects of TF, TR and EGCG against copper and cadmium-induced toxicity were observed in COS-7 cells. A 24-hr exposure to 500 μM copper resulted in 70~80% cell deaths. Pre-treatment with 10, 20, 40 or 80 μg/mL TF, TR and EGCG protected cells against the cytotoxic effects of copper. Exposure to 5 μM cadmium resulted in 50~60% cell deaths. Pre-treatment with 10, 20, 40 or 80 μg/mL TF, TR and EGCG protected cells against the cytotoxic effects of cadmium. Therefore, protective effects against the toxic affects of copper and cadmium transition metals were observed for TF, TR and EGCG (Tables 2 and 3).

Table 2. Effect of TF, TR and EGCG on copper-induced toxicity in COS-7 cells treated with antioxidants prior to a 24-hr exposure to 500 μM $CuSO_4$ on cell viability was determined

Sample amount (μg/mL)	Mean cell viability (%)		
	TF	TR	EGCG
0	35.1 ± 3.27^{Ac}	36.8 ± 4.26^{Ae}	24.5 ± 2.48^{Be}
10	59.6 ± 2.93^{ABb}	62.3 ± 2.09^{Ad}	56.1 ± 0.80^{Bd}
20	60.0 ± 2.25^{Cb}	68.7 ± 1.16^{Ac}	66.2 ± 1.29^{Bc}
40	69.9 ± 0.45^{Ca}	73.3 ± 1.21^{Bb}	84.9 ± 1.80^{Ab}
80	72.9 ± 0.88^{Ca}	81.8 ± 0.89^{Ba}	94.7 ± 1.08^{Aa}

The results are expressed as means±SD of triplicate assays. Means with different capital and small letters in superscripts within the same rows and columns are significantly different at $p<0.05$, respectively.

Table 3. Effect of TF, TR and EGCG on cadmium-induced toxicity, the effect of treating COS-7 cells with antioxidants prior to a 24-hr exposure to 5 μM $CdCl_2$ on cell viability was determined

Sample amount (μg/mL)	Mean cell viability (%)		
	TF	TR	EGCG
0	49.0 ± 1.55^{Cd}	59.7 ± 1.02^{Bc}	62.3 ± 1.15^{Ac}
10	47.1 ± 1.54^{Cd}	55.9 ± 0.71^{Bd}	63.5 ± 1.13^{Ac}
20	55.9 ± 0.28^{Bc}	54.8 ± 2.19^{Bd}	62.0 ± 0.86^{Ac}
40	63.4 ± 2.93^{Cb}	71.7 ± 0.79^{Bb}	80.5 ± 1.29^{Ab}
80	73.5 ± 4.83^{Ca}	86.9 ± 0.42^{Ba}	97.4 ± 0.68^{Aa}

The results are expressed as means±SD of triplicate assays. Means with different capital and small letters in superscripts within the same rows and columns are significantly different at $p<0.05$, respectively.

TF, TR and EGCG showed hydroxyl radical scavenging activity of about 62.0%, 50.9% and 77% at a concentration of 80 μg/mL. A concentration dependent inhibition of hydroxyl radical induced deoxyribose degradation was observed in the deoxyribose assays. In the β-carotene linoleate model system, TF, TR and EGCG at a concentration of 80 μg/mL were found to provide high antioxidant activity of 86.6, 79.7, and 80%, respectively in Fig. 1 and 2.

Differences in the degree of protection provided by TF, TR and EGCG against copper- and cadmium-induced toxicity were observed in this study. Cell death following copper exposure is partially attributable to the production of reactive oxygen species. In this study, copper toxicity was reduced by pre-treatment with TF, TR and EGCG. These results demonstrate that toxicity induced in endothelial cells by the Fenton catalyst iron

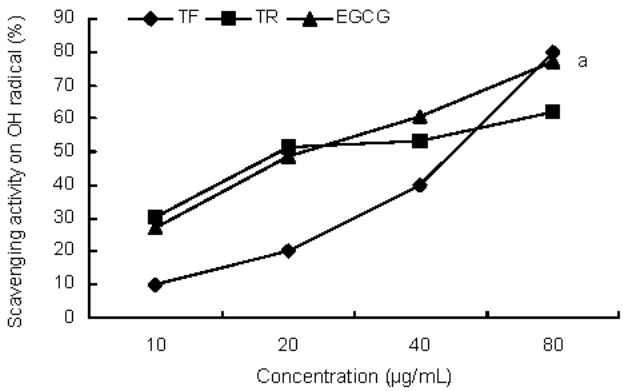

Fig. 1. The scavenging activity on hydroxyl radical of TF, TR and EGCG. [a]Data are presented as the mean±SD. Experiment was tested in triplicate for each dose of each sample.

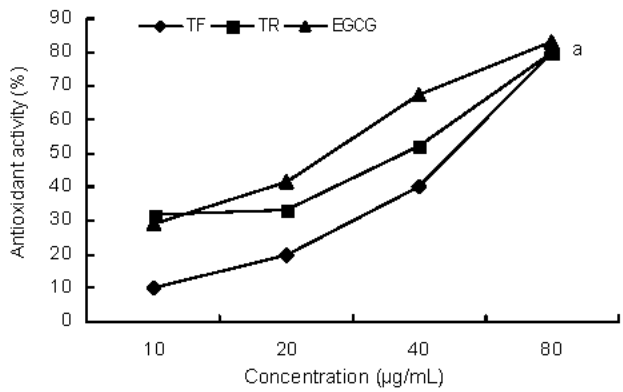

Fig. 2. Antioxidant activity of TF, TR and EGCG. [a]Data are presented as the mean±SD. Experiment was tested in triplicate for each dose of each sample.

was decreased by pre-treatment with TF, TR and EGCG. Previous studies have suggested that tea possess antioxidant and radical scavenging properties and metal-binding abilities (16). The sequestration of metals ions could result in decreases in the production of reactive oxygen species, oxidative damage and cytotoxicity (17). TF, TR and EGCG were also effective at attenuating cadmium toxicity, although not to the same extent as with copper. While cadmium induces oxidative stress as a secondary response, copper directly produced reactive oxygen species (18). A study by Romeo et al. (19) demonstrated differential effects of cadmium and copper on lipid per-oxidation and catalase activity in the kidney of sea bass. Reasons for the difference may be due to cell type, a function of exposure times to the metals or the duration of the preincubation. TF, TR and EGCG may well act as antioxidants and scavenge hydroxyl radicals generated from the Fenton reagent, even if the concentration changed, the inhibition curve was not largely shifted, i.e. compounds inhibited hydroxyl radicals generating system, rather than scavenging hydroxyl radicals. Therefore, hydroxyl radical scavenging activity was not due to direct scavenging but inhibition of hydroxyl radicals generation by transition metals such as iron (Fe^{2+}) or copper (Cu^{2+}) (14). The antioxidant activity of carotenoids is based on the radical adducts of carotenoids with free radicals from linoleic acid. The linoleic acid free radical attacks the highly unsaturated β-carotene models. The presence of carotenoid shows not only a decrease of the free radical concentration, but also the reduction of Fe^{3+} to Fe^{2+} by carotenoids. The presence of TF, TR and EGCG can hinder the extent of β-carotene – bleaching by neutralizing the linoleate-free radical and other free radicals formed in the system (20). Furthermore, it can be concluded that the Korean microbially fermented tea polyphenols, TF and TR show considerable antioxidant activity as observed in vitro in free radical scavenging activity and in kidney cells of monkey. Thus more detailed work is required on the antioxidant activity and protective effect of TF and TR.

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