Journal of Food and Drug Analysis, Vol. 12, No. 3, 2004, Pages 277-285 藥物食品分析 第十二卷 第三期

Aqueous Extract from Taiwanese *Agrocybe cylindracea* strain B Protects DNA against ·OH- mediated Strand Breaks

LIEN-HUI WANG[1], SHUN-JEN TSAI[2] AND SHWU-YUANN LIN[3]*

[1.] *Department of Food Science and Technology, Chung Hwa College of Medical Technology, 89 Wunhua 1st St., Rende Township, Tainan County 717, Taiwan, R.O.C.*

[2.] *Department of Food Science, National Chung Hsing University, 250 Guoguang Rd., South District, Taichung City 402, Taiwan, R.O.C.*

[3.] *Department of Food and Nutrition, Chung Hwa College of Medical Technology, 89 Wunhua 1st St., Rende Township, Tainan County 717, Taiwan, R.O.C.*

(Received: August 20, 2003: Accepted: July 8, 2004)

ABSTRACT

Water extract from *Agrocybe cylindracea* strain B (ACE), an edible mushroom produced in Taiwan, has previously been studied for its antioxidative activities. ACE is an efficient antioxidant *in vitro*. Since oxidative alterations of DNA can be linked to the development of cancer, we decided to study whether ACE protects DNA against oxidative stress. We report here that pUC18 plasmid DNA and HepG2 cells' DNA are damaged by • OH radicals generated from Cu (II) plus H_2O_2. The DNA damage was quantified by determining the diminution of supercoiled DNA forms and the DNA migration after oxidative attack using agarose gel electrophoresis. The IC_{50} values of ACE were 69.7 μg/mL and 91.6 μg/mL for cupric-mediated • OH formation by determining the diminution of supercoiled DNA forms and Comet assay, respectively. The antioxidant capacity of ACE was significantly higher than that of *Lentinus edodes* extract (LEE) and the two known scavengers of hydroxyl radical, mannitol and thiourea. ACE and LEE could also inhibit the oxidative degradation of 2'-deoxyribose caused by • OH radicals generated from metal ions plus H_2O_2. The malondialdehyde (MDA) inhibition effect of active fractional protein from ACE was better than that of LEE. The major protein components of ACE were different from LEE.

Key words: *Agrocybe cylindracea*, DNA damage, hydroxyl radical, antioxidative activity

INTRODUCTION

Oxidative DNA damage by reactive oxygen species (ROS) has been hypothesized to play a critical role in several diverse biological processes including mutagenesis, carcinogenesis, aging, radiation damage, and cancer chemotherapy[1-2]. Cellular metabolism has been shown to generate oxygen species such as hydrogen peroxide (H_2O_2), hydroxyl radical (· OH), singlet oxygen ($^1O_2$), superoxide anion (O_2^- ·), and hydroperoxyl radical[3]. They attack biological molecules and induced damage to cell or tissue injury[4].

The limitation of synthetic antioxidants such as BHA, BHT, TBHQ and others have been shown in reviews of toxicity, cancer and promutagenic activity. This has resulted in increased demand for natural antioxidants. Much attention has been focused on antioxidant mechanism from natural sources[5]. Recently, research on phytochemicals in food materials and their effects on health, especially the suppression of active oxygen species by natural antioxidants from teas[6], spices[7], and mushrooms[8-11], had been intensively studied. It has been shown that certain species of mushrooms have the capacity to lower plasma cholesterol level[12-14] and blood pressure[15], antitumor activity[16-17] and antidiabetic activity[18]. Also, antioxidant and free radical scavenging activities of mushrooms and their constituents have been demonstrated using *in vitro* systems[5,19-22].

Agrocybe cylindracea (DC: Fr.) Mre. [syn. Agrocybe cylindracea (Briganti) Singer] strain B is a newly cultured edible mushroom in Taiwan[22]. It was introduced from Japan into Taiwan by Lung-Kuo Mushroom Farm, Taichung, since 1986[22]. It has been reported that *A. cylindracea* had antitumor activity[23] and free radical scavenging activity[24]. We recently demonstrated that water extract from *A. cylindracea* strain B (ACE) has antioxidant activity against iron-mediated lipid peroxidation of liposome[25]. However, a few data are available about the protective activity of ACE on DNA damage induced by ROS. In the present study investigates the protection effects of ACE and its protein fraction on DNA damage induced by ROS. The water extract of shiitake mushroom *L. edodes* (LEE) used as a control for comparison of protection effect of ACE was also investigated.

* Author for correspondence. Tel: +886-6-2605779; Fax: +886-6-2605779; E-mail: linshwnyuann@yahoo.com.tw

MATERIALS AND METHODS

I. Preparation of Mushroom Extracts

The strain B of *A. cylindracea* was cultivated at Lung-Kuo Mushroom Cultivation Farm, (Taichung, Taiwan). *L. edodes* was purchased at a supermarket in Taichung. Mushrooms were harvested before the veils broke, and sliced and ground with Waring Blendor. Then the antioxidants were extracted as follows: 200 g of mushroom was extracted with 400 mL of deionized water for 30 min, and then centrifugated at 12,000 rpm for 30 min at 4˚C. All extracts were filtered using Whatman No. 1 filter paper. Extraction was repeated twice, and the combined filtrates were dried with lyophilizer.

II. *Assay of DNA Breakage*

DNA breaks were detected using an adaptation of the method described above[2]. Supercoiled plasmid DNA (pUC18, 0.1 μg) in 0.145 M NaCl, 0.01 M sodium phosphate, pH 7.0, was incubated for 1 hr at 37˚C with 10 μM $CuSO_4$ / 1 mM H_2O_2 and /or mushroom extracts in a total volume of 4 μL. DNA damage occurred with 250 μM H_2O_2 plus 2.5 μM Cu (II). The reaction was stopped by addition of 1 μL of 0.5 M EDTA (pH 8.0). One microliter of loading buffer [0.025% bromophenol blue, 0.025% xylene cyanole, 40% sucrose in 0.4 M Tris, 0.2 M sodium acetate, 0.02 M EDTA, pH 7.8 (10 × TAE buffer)] was added and the samples were analyzed by carrying out electrophoresis for 60 min at 50 V in 1% agarose in 1 × TAE buffer. The gel was stained with ethidium bromide and photographed.

III. *Electron Paramagnetic Resonance (EPR) Spectrometry*

The hydroxyl radical was detected using an adaptation of the method previously described [26]. The hydroxyl radical rapidly reacts with the nitrone spin trap 5,5-dimethyl pyrrolidine–N–oxide(DMPO) and the resultant DMPO-OH adduct was detected with an Electron Paramagnetic Resonance (EPR) spectrometry. The EPR spectrum was recorded for 2.5 min after mixing sample with phosphate buffer (0.1 M, pH 7.4), 10 mM EDTA, 10 mM H_2O_2, 0.3 M DMPO and 5 mM $FeSO_4$ using an EPR spectrometer (Bruker EMX-10/12, Germany) set at the following conditions: 3480-G magnetic field, 1.0 G modulation amplitude, 0.5 S time constant and 200 S scan period.

IV. *Cell Culture*

The HepG2 cells were obtained from American Type Culture Collection (ATCC). The cells were routinely grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco BRL) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco BRL), 2 mM glutamine, 10 mM non-essential amino acid, 100 mM sodium pyruvate solution, 5.5% bicarbonate, 100 U/mL penicillin and 100 μg/mL streptomycin. The cultures were incubated at 37˚C in a humidified atmosphere containing 5% CO_2. The cells were subcultured every 3 to 4 days.

V. *$CuSO_4$ / H_2O_2-induced DNA Damage in HepG2 Cells*

To induce DNA damage, HepG2 cells (5×10^5) grown in 5 cm dishes were exposed to 10 μM $CuSO_4$ / 1 mM H_2O_2 in the presence or absence of mushroom extracts (0.05, 0.1 or 0.2 mg/mL) in DMEM medium for 2 hr at 37˚C in 5% CO_2 / 95% air. The cells were washed twice with phosphate buffer saline (1 × PBS), pH 7.4, and trypsinized. The cells were centrifugated at 3,500 rpm for 5 min at 4˚C and suspended in low melting point agarose (1%) for Comet assay.

V. *Single Cell Gel Electrophoresis Assay (Comet Assay)*

DNA breaks were detected using an adaptation of the method previously described[27]. HepG2 cells treated with 10 μM $CuSO_4$ / 1 mM H_2O_2 and / or mushroom extract were suspended in 80 μL 1% (w/v) low melting point agarose in PBS (pH 7.4) at 37˚C. The treated cells were immediately pipetted onto frosted glass microscope slides precoated with a layer of 1% (w/v) normal melting point agarose similarly prepared in PBS. The agarose was allowed to set for 10 min on ice and then the slides were immersed in lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, 1% sodium lauroylsarcosinate, pH 10.0 with NaOH, 10% v/v dimethyl sulfoxide and 1% v/v Triton X-100) at 4˚C for 1 hr to remove cellular proteins. Slides were then placed in a horizontal electrophoresis tank containing electrophoresis buffer (300 mM NaOH and 1 mM Na_2EDTA) for 15 min before electrophoresis at 25 V, 300 mA for 25 min. Then the slides were washed 3 times for 5 min each with neutralizing solution (0.4 M Tris-HCl, pH 7.5) at room temperature before they were stained with 20 μg/mL ethidium bromide. Ethidium bromide-stained slides were examined under a Nikon Eclipse TE-200 fluorescence microscope (Nikon, Japan). Fifty comets on 3 slides were quantified by computerized image analysis Inspectrox 2.1 (Matrox Electronic System Ltd., Canada) on the basis of DNA migration (tail length). The untreated HepG2 cells (blank control) and cells treated with benzo[a]pyrene (BaP), a known inducer of DNA migration, were included in each Comet assay. The DNA migrations of blank controls were very low. This showed that no DNA damage was caused by the comet assay. Each data represents the means ± SD of three separate experiments.

VI. *ACE Fraction and SDS-PAGE Analysis*

The fraction analysis was performed according to the method previously described[28]. Mushroom extract protein was isolated as follows: the ACE solution was applied to a carboxymethyl-cellulose (CM cellulose) column (2.2 × 30

cm) which was previously equilibrated with 10 mM sodium acetate (pH 5.2). The column was first washed with 10 mM equilibration buffer and then eluted with 100mL of 0-0.5N NaCl in 10 mM sodium acetate (pH 5.2). The LEE solution was applied to a DEAE - cellulose column (2.2 × 30 cm) which was previously equilibrated with 10 mM phosphate buffer (pH 7.8). The column was first washed with 10 mM equilibration buffer and then eluted with 100 mL of 0-0.5N NaCl in 10 mM phosphate buffer (pH 7.8). Collection was eluted by fraction collector at a rate of 1 tube / min and then detected protein content and total carbohydrates with spectrophotometer at 280 nm and 490 nm, according to the ultraviolet absorption assay[29] and the phenol-sulfuric acid method[30], respectively. Mushroom protein fractions and crude extracts were resolved using SDS-PAGE with 10% polyacrylamide gel at 100 V for 100 min on a Hoefer mini slab gel apparatus. The gel was then stained with Coomassie brilliant blue R solution. Its antioxidative activity was measured by TBA method as described in the following section.

VII. *Determination of Damage to 2'-Deoxyribose*

The inhibition effect on oxidative degradation of 2'-deoxyribose was determined by the thiobarbituric acid (TBA) methods[31-32] after incubating the reaction mixture for 1 hr at 37°C. Hydroxyl radical was generated by a mixture of ascorbic acid, H_2O_2 and metal ions–EDTA, attacking 2'-deoxyribose to degrade into fragments that give rise to a chromogen upon heating with thiobarbituric acid under acidic condition. Therefore, 1 mM 2'-deoxyribose in 0.1 M sodium phosphate (pH 7.4) was incubated for 1 hr at 37°C with 10 μM EDTA, 10 μM ascorbic acid, 1 mM H_2O_2, 10 μM Cu^{2+} and / or ACE, LEE, other scavengers and mushroom protein fractions in a total volume of 1 mL. The reaction was stopped by addition of 0.1 mL of 20 mg/mL BHT. Damage to 2'-deoxyribose caused by H_2O_2 plus Cu (II) (in 100 mM PBS buffer, pH 7.4) was determined by quantification of TBARs at 532 nm. All test data are presented as an average of triplicate analyses.

VIII. *Statistical Analysis*

Results are expressed as the mean ± SD, with n = 3. Statistical evaluations were performed by analysis of variance followed by Duncan's test for dose-response data; $p < 0.05$ was considered significant.

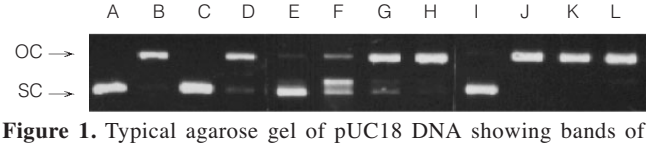

Figure 1. Typical agarose gel of pUC18 DNA showing bands of supercoiled (SC) and open circular (OC) forms. Experimental conditions are as described in Materials and Methods. Lanes on the gel represent: (A) control (no H_2O_2 or Cu^{2+}), (B) 250 μM H_2O_2 +2.5 μM Cu^{2+}, (C) 2.5 mM mannitol alone, (D) 250 μM H_2O_2 +2.5 μM Cu^{2+} plus 2.5 mM mannitol, (E) 62.5 μg/mL ACE alone, (F-H) 250 μM H_2O_2 +2.5 μM Cu^{2+} plus ACE (F) 62.5, (G) 25.0, or (H) 12.5 μg/mL, (I) 62.5 μg/mL LEE alone, (J-l) 250 μM H_2O_2 +2.5 μM Cu^{2+} plus LEE (J) 62.5, (K) 25.0, or (L) 12.5 μg/mL. Abbreviations: ACE, water-extract from *A. cylindracea*. LEE, water-extract from *L. edodes*.

RESULTS

I. *Preparation of Mushroom Extracts*

All extracts were combined and dried with lyophilizer. The yields of powder of ACE and LEE were 44.13% (dry basis) and 42.35% (dry basis), respectively.

II. *Prevention of DNA Damage by ACE and Other Scavengers*

To understand whether ACE could prevent DNA damage induced by oxidative stress, another known scavenger, mannitol, was selected as positive control. As shown in Figure 1, control preparations of pUC18

Table 1. Protection against oxidative damage to pUC18 by ACE and other chelators

Treatment	SC DNA(%)[a]	Protection(%)[b]	IC_{50} (μg/mL)
Solvent control	88.75 ± 3.38	100	
250 μM H_2O_2 / 2.5 μM $CuSO_4$	47.91 ± 3.02	0	
+ ACE 62.5 μg/mL	65.45 ± 6.79*	42.95	
+ ACE 25.0 μg/mL	55.52 ± 2.05*	18.65	69.7
+ ACE 12.5 μg/mL	46.05 ± 4.13	0	
+ LEE 62.5 μg/mL	54.31 ± 14.22	15.67	
+ LEE 25.0 μg/mL	53.36 ± 16.48	13.35	326.18
+ LEE 12.5 μg/mL	51.28 ± 14.67	8.26	
+ mannitol 2.5 mM	56.34 ± 2.08*	20.64	
mannitol 2.5 mM alone	87.15 ± 3.88	–	
ACE 62.5 μg/mL alone	86.03 ± 4.68	–	
LEE 62.5 μg/mL alone	85.42 ± 3.21	–	

[a]Experimental conditions are as described in Materials and Methods. Values are means ± SD (n = 3). SC DNA (%) = percentage of supercoiled form DNA.

[b]The % protection was calculated as: % protection = 100 × [1 - (% SC of control -% SC of scavenger) / (% SC of control - % SC of H_2O_2 / Cu (II))].

*Significantly different from positive control at $p \leq 0.05$. Abbreviations are the same as Figure 1.

Table 2. Effect of ACE and LEE on EPR signal intensity of DMPO-OH spin adducts

Sample	Relative EPR signal intensity[a] (IP %[b]) Extract dose (mg/mL)			IC_{50} (μg/mL)
	0.1	0.5	2.0	
Control	100	100	100	
Mannitol	–	–	51.3 ± 0.2*(49)	N.D.
Thiourea	–	32.3 ± 0.4*(68)	–	N.D.
ACE	49.0 ± 0.5*(51)	8.2 ± 0.6*(92)	3.2 ± 0.3*(97)	< 100
LEE	105.5 ± 0.8 (-5)	86.0 ± 0.4*(14)	53.1 ± 0.6*(47)	> 2000

[a]Experimental conditions are as described in Materials and Methods. Relative EPR signal intensity (%) = {[h ΔH2(sample) / h ΔH2 (dpph)] / [h ΔH2 (control) / h ΔH2 (dpph)]} × 100. h = the width of the peak, ΔH = the length of the peak stress. Values are means % ± SD (n = 3).
[b]IP (%) = Percentage of inhibition = [relative EPR signal intensity of solvent control- relative EPR signal intensity of treatment]
*Significantly lower than solvent control at $p \leq 0.05$. Each value is the means of 4 peaks. Abbreviations are the same as Figure 1.

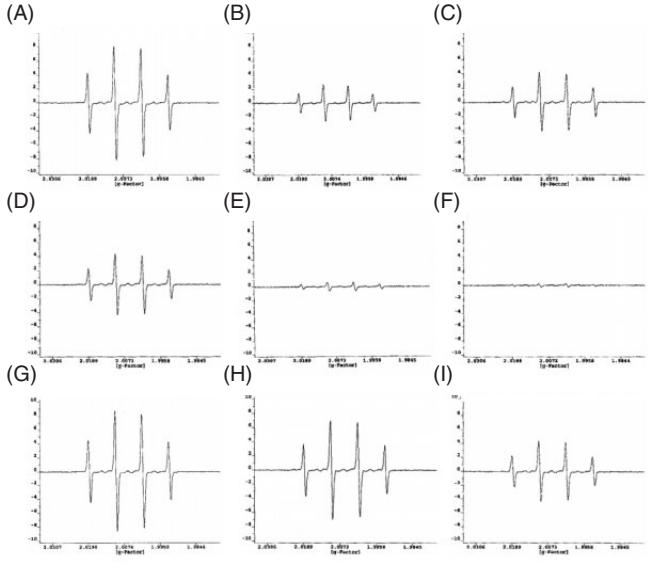

Figure 2. Typical EPR signal of DMPO-OH spin adduct. (A) control; (B) 0.5 mg/mL thiourea; (C) 2.0 mg/mL mannitol; (D-F) 0.1, 0.5 and 2.0 mg/mL of ACE, respectively; (G-I) 0.1, 0.5 and 2.0 mg/mL of LEE, respectively. Abbreviations are the same as Figure 1.

contained 88.75 ± 3.38% supercoiled (SC) DNA (lane A), and the levels of which were not affected by incubation with 2.5 mM mannitol, 62.5 μg/mL ACE or LEE for 1 hr (lane C, E and I, respectively). ACE at a concentration of 62.5 μg/mL and 25 μg/mL protected pUC18 DNA from damage while hydroxyl radicals were generated by 2.5 μM Cu (II) plus 250 μM H_2O_2 (lane F-G). However, addition of increasing concentrations of ACE caused the protection effect on breakage of pUC18. More protection occurred with ACE than LEE under the same concentrations. A quantitative comparison of the protective effect of ACE or LEE against the DNA damage induced by · OH–mediated reagents is shown in Table 1. IC_{50} is the concentration of scavengers required to inhibit the DNA damage of oxidative stress by 50%. These values were derived from extrapolation of the dose-response curves. Although all scavengers had little efficiency in protecting against DNA damage, ACE was more effective than LEE. On the basis of IC_{50}, the protection effect of ACE was 5–fold greater than that of LEE.

Table 3. Effect of ACE and other chelators on the 2'-deoxyribose damage induced by H_2O_2/ Cu^{2+}

Treatment	OD 532 nm ± SD	(IP %[a])	IC_{50} (μg/mL)
Solvent control	0.661 ± 0.009	(0.0)	---
ACE			
2000 μg/mL	0.033 ± 0.005*	(95.0)	
500 μg/mL	0.077 ± 0.004*	(88.4)	
100 μg/mL	0.223 ± 0.026*	(66.3)	49.12
LEE			
2000 μg/mL	0.034 ± 0.009*	(94.8)	
500 μg/mL	0.202 ± 0.007*	(69.5)	
100 μg/mL	0.461 ± 0.009*	(30.3)	358.26
Mannitol			
2000 μg/mL	0.329 ± 0.015*	(50.3)	
500 μg/mL	0.422 ± 0.007*	(36.1)	
100 μg/mL	0.520 ± 0.006*	(21.4)	1903.31
Thiourea			
2000 μg/mL	0.068 ± 0.007*	(89.7)	
500 μg/mL	0.135 ± 0.008*	(79.6)	
100 μg/mL	0.457 ± 0.005*	(30.9)	256.77

[a]IP (%): inhibition percentage = [1-(OD_{532} of treatment/ OD_{532} of solvent control)] × 100%. 2-Deoxyribose was treated with H_2O_2/Cu(II) plus various concentrations of ACE or LEE at 37°C for 1 hr. Abbreviations are the same as Figure 1.
*Significantly lower than solvent control at $p \leq 0.05$. The mean ± SD is given for 3 independent experiments.

II. *ACE Prevents the Formation of Hydroxyl Radicals*

We demonstrated that ACE inhibits oxidative DNA damage caused by Cu (II) plus H_2O_2. The EPR studies using DMPO as a spin trap indicated that ACE can inhibit the generation of · OH radicals *via* the Fenton reaction. ACE had better inhibition effect than LEE and thiourea (Table 2 and Figure 2). As shown in Table 2, 0.5 mg/mL ACE inhibited 92% of DMPO-OH adduct caused by Fe (II) plus H_2O_2, while only 14% and 68% of DMPO-OH adduct was inhibited by LEE and thiourea under the same concentration of 0.5 mg/mL, respectively.

ACE and LEE could also significantly inhibit the oxidative degradation of 2'-deoxyribose caused by · OH radicals generated from Cu (II) plus H_2O_2. The inhibition effect on oxidative degradation of 2'-deoxyribose caused by · OH radicals is shown in Table 3. IC_{50} values were derived from interpolation and extrapolation of the dose-

Journal of Food and Drug Analysis, Vol. 12, No. 3, 2004

response curves. IC_{50} of ACE was 49 μg/mL which was lower than that of LEE (358 μg/mL), thiourea (257 μg/mL) and mannitol (1903 μg/mL). On the basis of IC_{50}, the protection effect of ACE was 7-, 5- and 38-fold greater than that of LEE, thiourea and mannitol, respectively.

III. *Effect of ACE on DNA Strand Breakage in HepG2 Cells*

We also investigated the inhibitory effect of ACE in HepG2 cells on Cu (II)/ H_2O_2-induced DNA damage using the single cell electrophoresis (Comet) assay. The DNA migrations of blank controls were the same as the solvent controls. This showed that no DNA damage was caused by Comet assay. As shown in Figure 3, ACE (200 μg/mL) significantly inhibited 10 μM Cu (II)/ 1 mM H_2O_2-induced DNA damage (Figure 3F) and did not itself produce DNA damage (Figure 3E). A quantitative comparison of the protective effect of ACE or LEE against the DNA damage induced by · OH–mediated reagents is shown in Table 4. ACE (200 μg/mL) inhibited 67% of DNA damage caused by Cu (II) plus H_2O_2 in HepG2 cells, while only 47% of DNA damage was inhibited by LEE under the same conditions employed as shown in Table 4. IC_{50} is the concentration of scavengers required to inhibit the DNA damage of oxidative stress by 50%. These values were derived from interpolation and extrapolation of the dose-response curves. IC_{50} of ACE was 91.58 μg/mL which was lower than that of LEE (214.14 μg/mL). Based on the IC_{50}, the protection effect of ACE was 2.3-fold greater than that of LEE.

IV. *Effect of ACE Fractions on Oxidation of 2'-Deoxyribose*

To further verify whether the inhibition activity of ACE on oxidative DNA damage induced by hydroxyl radicals is mediated through protein or other carbohydrates, ACE was fractioned with CM-cellulose column. The fraction profiles(such as protein content and total carbohy-

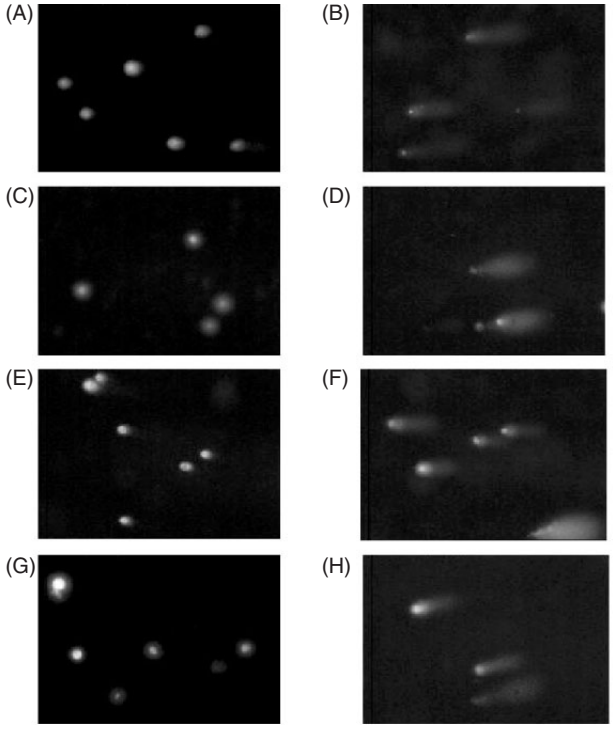

Figure 3. Representative comet images of HepG2 cells following treatment with mushroom extracts and/or 1 mM H_2O_2 plus 10 μM Cu (II). Cells were treated with sample with or without H_2O_2 plus Cu (II) at 37°C for 2 hr. (A) DMSO; (B) Bap 20 μM; (C) control; (D) 1 mM H_2O_2 +10 μM Cu(II); (E) ACE 200 μg/mL alone; (F) 1 mM H_2O_2 + 10 mM Cu(II) + ACE 200 μg/mL; (G) LEE 200 μg/mL alone; (H) 1 mM H_2O_2 + 10 μM Cu(II) + LEE 200 μg/mL. Abbreviations are the same as Figure 1.

Table 4. Protection effect of ACE and LEE on DNA migration induced by oxidative stress in HepG2 cells

Treatment	DNA migration[a] ± SD (μm)	Percent of grade[b]					Protection[c] (%)	IC_{50} (μg/mL)
		1	2	3	4	5		
Blank	19.04 ± 6.34	64	36	0	0	0		
1 mM H_2O_2 /10 μM $CuSO_4$	80.37 ± 19.42	0	0	0	32	68		
+ ACE 200 μg/mL	39.43 ± 9.74*	5	38	57	0	0	66.75	91.58
+ ACE 100 μg/mL	49.26 ± 9.76*	1	15	74	10	0	50.72	
+ ACE 50 μg/mL	62.76 ± 6.34	0	2	33	65	0	28.97	
+ LEE 200 μg/mL	51.44 ± 5.76*	0	3	92	5	0	47.17	214.14
+ LEE 100 μg/mL	55.02 ± 4.61*	0	0	89	11	0	41.33	
+ LEE 50 μg/mL	62.31 ± 7.46	0	0	45	54	1	29.45	
ACE 200 μg/mL alone	25.39 ± 10.9	48	50	2	0	0		
LEE 200 μg/mL alone	29.27 ± 13.37	37	42	21	0	0		
DMSO (solvent control)	16.69 ± 8.97	77	20	0	0	0		
Bap 20 μM	70.22 ± 4.53	0	0	5	93	2		

[a]The average DNA migration (DM) ± SD is given for 3 independent experiments.

[b]The grade of DNA migration as following: Grade 1 = 0~20 μm, Grade 2 = 21~40 μm, Grade 3 = 41~60 μm, Grade 4 = 61~80 μm, Grade 5 > 80 μm.

[c]The percent of protection was calculated as: protection (%) =100 × [(DM of H_2O_2 /Cu (II) – DM of scavenger) / (DM of H_2O_2/Cu (II) – DM of blank)].

*Significantly lower than H_2O_2/Cu(II) treatment at $p \leq 0.05$. Cells were treated with H_2O_2/Cu (II) plus various concentrations of ACE or LEE at 37°C for 2 hr. Abbreviations are the same as Table 1.

drate content) and their inhibition properties were detected with SDS-PAGE electrophoresis, phenol-sulfuric acid and 2'-deoxyribose method, respectively. As shown in Figure 4, the protein contents were higher between ACE fraction No.1-20 and No.80-110, whereas the total carbohydrate contents were higher between ACE fraction No.1-20 and No.56-63. However, the inhibition properties of ACE fraction were higher between No.1-20, No.55-70 and No.80-110, which were 74.2%, 49.8% and 54.9%, respectively. Moreover, the MDA inhibition effect of fraction No. 80-110 was better than that of fraction No. 1-20 under the same concentration. Therefore, we hypothesized the protein fraction to be the major active compound of ACE, which may not be similar as LEE. The major active proteins of ACE or LEE are shown in Figure 5. The molecular mass of the antioxidative proteins in ACE was estimated to be about 43 kDa and 29 kDa by SDS-PAGE (Figure 5, lane C), which were different from those in LEE (Figure 5, lane B). The protein fractions from ACE or LEE could also inhibit the oxidative degradation of 2'-deoxyribose caused by · OH radicals generated from Cu (II) plus H_2O_2 (Figure 6). The antioxidative capacity of ACE protein fraction was significantly higher than LEE fraction under the same concentration (Figure 6).

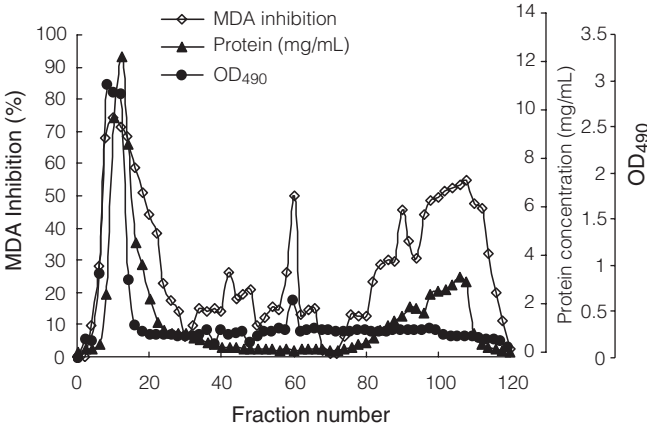

Figure 4. The MDA inhibition effect of ACE CM-fractions on oxidative degradation of 2'-deoxyribose induced by hydroxyl radicals and its protein (mg/mL) or total carbohydrate concentrations (OD_{490}). Abbreviations are the same as Figure 1.

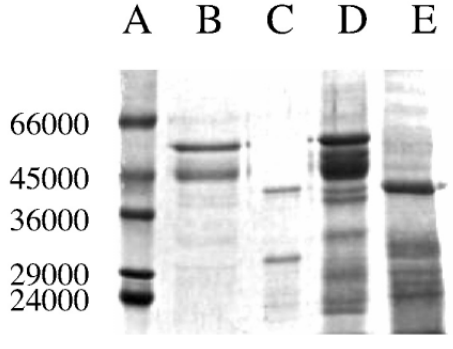

Figure 5. Electrophoretic analysis of active protein from CM fraction of ACE and DEAE fraction of LEE. Lane A: molecular mass markers from Sigma: (66 kDa), (45 kDa), (36 kDa), (29 kDa) and (24 kDa); Lane B: active fraction from LEE (LE 100) with DEAE-cellulose column chromatography; Lane C: active fraction from ACE (AC 107) with CM-cellulose column chromatography; Lane D: LEE (25 mg/mL), Lane E: ACE (25 mg/mL). Abbreviations are the same as Figure 1.

DISCUSSION

Active oxygen species may be involved in the etiology of diverse human diseases, such as coronary heart disease, stroke, rheumatoid arthritis and cancer[33-34]. The limitation of synthetic antioxidants such as BHA, BHT, and TBHQ, etc. have been shown in reviews of toxicity, cancer and promutagenic activity. This would result in increased demand for natural antioxidants. Much attention has been focused on antioxidant mechanism from natural sources[5]. Trace metals such as copper and iron may interact with active oxygen species, ionizing radiation, or microwaves to damage DNA[2]. Iron and copper are ubiquitous metals in cells, as well as many enzymes and proteins. Their ionic forms are prone to participate in one-electron transfer reactions, and this is one important attribute for their use as prosthetic groups in enzyme that catalyze redox reactions. However, this capacity enables iron and copper to generate radical species as well. For example, copper and iron participate in the Fenton reaction, in which the reactive hydroxyl radical is generated:

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + \cdot OH + OH^-$$

The rate constant for the Fenton reaction is higher for copper than for iron[35]. The hydroxyl radical is an extremely reactive free radical formed in biological systems. It reacts rapidly with almost every molecule in living cells, such as sugars, amino acids, DNA bases and organic acids. An example of a detector for hydroxyl radical is the sugar 2'-deoxy-D-ribose[32], which is also a component of DNA.

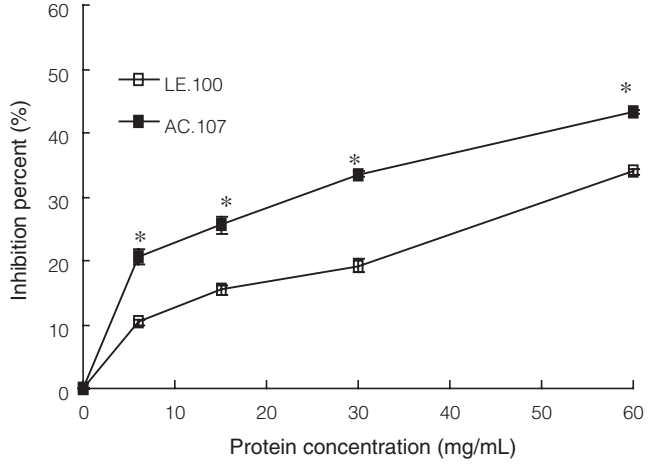

Figure 6. The inhibition activity of AC107 and LE100 on oxidative degradation of 2'-deoxyribose induced by hydroxyl radicals. *Significantly higher than *L. edodes* at $p \leq 0.05$. Abbreviations are the same as Figure 1.

In the "deoxyribose method", the hydroxyl radical was generated by a mixture of ascorbic acid, H_2O_2 and metal ions–EDTA, attacking deoxyribose to degrade into fragments that give rise to a chromogen upon heating with thiobarbituric acid[36-37] under acidic condition.

A variety of compounds from mushrooms have been found as antioxidants, including polysaccharides[38], melanin[39-40], SOD-mimic compounds[41], SOD[42-43], and triterpene[44], which have a direct effect on free radical scavenging activities. In a previous report, several extracts of mushrooms prepared by using ethanol, particularly those from the mushroom, *Suillus bovinus(L.: Fr.) O. Kuntze*, were shown to have strong antioxidative activity[5,19]. Moreover, it has been demonstrated that methanol extract of *A. cylindracea* contained two indole extracted derivatives that had free radical scavenging activities[24].

The extract from *A. cylindracea* strain B, an edible mushroom produced in Taiwan, has previously been studied for its antioxidative activities. It is an efficient *in vitro* antioxidant. Our previous study has shown that ACE inhibit lipid peroxidation caused by Fe^{3+}/H_2O_2/ascorbate, and the antioxidant activity of ACE may also be due to its scavenging activity on the hydroxyl radical[25]. In this study, the protection effect of ACE on DNA damage was attributed to its capacity to scavenge free radicals, especially on hydroxyl radical. As shown in Table 3, the protective capacity of ACE was significantly higher than that of LEE.

It has been reported that mushrooms contain substances with chemical properties similar to ascorbic acid[45]. Our preliminary data indicated that ACE has less reducing capacity than LEE (data not shown), which cannot fully explain the higher scavenging activity of ACE. Since tocopherol contents of mushrooms were approximately 150 μg/100 g dry matter, it seemed that tocopherol was not the main antioxidant component of mushrooms[19]. Mushroom polysaccharide extracts had superoxide and hydroxyl radical scavenging activities[38], but polysaccharide was less dissolved in cool water. Moreover, the two indole derivatives of *A. cylindracea* were extracted with methanol. Therefore, we hypothesize that the protein components of ACE appeared to contribute to free radical scavenging activity[38]. It had been demonstrated that 107 KDa protein dimer, extract from shiitake mushroom, has scavenging activity and protection effect on DNA damage induced by ROS. We also found that the water-soluble protein isolated from the fruit body of *A. cylindracea* was as effective in protecting against hydroxyl radical-induced oxidation of 2'-deoxyribose (Figure 6), indicating that the protein compound is associated with the protective properties. We hypothesized that the protein profile of ACE was different from that of LEE. According to the result of SDS-PAGE as shown in Figure 5, we demonstrated that the active proteins of ACE were different from that of LEE.

Using agarose gel electrophoresis, we have evaluated the potential of ACE as an antioxidant defense against oxygen radical-mediated damage. Although the evidence presented here is based on *in vitro* experiment using isolated DNA, the results clearly demonstrate that the water extract of *A. cylindracea* shows good antioxidative activity, as well as protection against DNA damage induced by metal-catalyzed Fenton reactions. As shown in Figure 6, the results indicated that fractional protein of ACE exhibited rapidly at a concentration-dependent increase in hydroxyl radical scavenging activity up to a concentration of 60 μg/mL. The scavenging activity was 44.6% at a concentration of 60 μg/mL ACE fractional protein. The hydroxyl radical scavenging activity of fractional protein from ACE was significantly stronger than that of LEE and also that of methanol extract of *G. tsugae*, which scavenged 53.4% of hydroxyl radical at a higher concentration of 5000 μg/mL[11].

According to the results described above, we suggest that edible *A. cylindracea* may be a potential chemoprevention foodstuff against oxidative stress. Therefore, further studies are needed to compare the activity of different solvent extracts from *A. cylindracea* and to identify the active component, and to study the protective mechanisms at the molecular level.

ACKNOWLEDGMENTS

We thank the chief of Lung-Kuo Mushroom Cultivation Farm, (Taichung, Taiwan) for providing the *A. cylindracea* mushroom samples.

REFERENCES

1. Ames, B. N. 1983. Dietary carcinogens and anticarcinogens: oxygen radicals and degenerative disease. Science 221: 1256-1264.
2. Sagripant, J. L. and Kraemer, K. H. 1989. Site specific oxidative DNA damage at polyguanosines produced by copper plus hydrogen peroxide. J. Biol. Chem. 264: 1729-1734.
3. Cerruti, P. A. 1985. Science 227: 375-381.
4. Niki, E. 1992. Active oxygens and free radicals in biology. J. Jap. Oil Chem. Soci. 41: 768-773.
5. Kasuga, A., Aoyagi, Y. and Sugahara, T. 1995. Antioxidant activity of fungus *Suillus bovinus (L:Fr.) O. kuntze*. J. Food Sci. 60: 1113-1115.
6. Grinberg, L. N., Newmark, H., Kitrossky, N., Rahamim, E., Chevion, M. and Rachmilewitz, E. A. 1997. Protective effects of tea polyphenols against oxidative damage to red blood cells. Biochem. Pharm. 54: 973-978.
7. Srinivas, L., Shalini, V. K. and Shylaja, M. 1992. Turmerin: a water soluble antioxidant peptide from Turmeric [*Curcuma longa*]. Arch. Biochem. Biophys. 292: 617-623.
8. Kim, H. S., Kacew, S. and Lee, B. M. 1999. *In vitro* chemopreventive effects of plant polysaccharides (*Aloe barbadensis Miller, Lentinus edodes, Ganoderma*

lucidum and Coriolus versicolor). Carcinogensis 20: 1637-1640.

9. Matsuzawa, T., Sano, M., Tomita, I., Saitoh, H. and Ikekawa, T. 1997. Studies on antioxidant effects of *Hypsizigus marmoreus*. I. Effects of *Hypsizigus marmoreus* for antioxidant activities of mice plasma. Yakugaku Zasshi- J. Pharm. Soc. Jap. 117: 623-628.
10. Matsuzawa, T., Saitoh, H., Sano, M., Tomita, I., Ohkawa, M. and Ikekawa, T. 1998. Studies on antioxidant effects of *Hypsizigus marmoreus*. II. Effects of *Hypsizigus marmoreus* for antioxidant activities of tumor-bearing mice. Yakugaku Zasshi- Journal of the Pharmaceutical Society of Japan 118: 476-481.
11. Yen, G. C. and Wu, J. Y. 1999. Antioxidant and radical scavenging properties of extracts from *Ganoderma tsugae*. Food Chem. 65: 375-379.
12. Bobek, P., Hromadova, M. and Ozdin, L. 1995. Oyster mushroom (*Pleurotus ostreatus*) reduces the activity of 3-hydroxy-3- methylglutaryl CoA reductase in rat liver microsomes. Experientia 51: 589-591.
13. Sugiyama, K., Akachi, T. and Yamakawa, A. 1993. The hypocholesterolemic action of *Lentinus edodes* is evoked through alteration of phospholipid composition of liver microsomes in rats. Biosci. Biotech. Biochem. 57: 1983-1985.
14. Sugiyama, K., Akachi, T. and Yamakawa, A. 1995. Eritadenine- induced alteration of hepatic phospholipid metabolism in relation to its hypocholesterolemic action in rats. J. Nutr. Biochem. 6: 80-87.
15. Adachi, K., Nanba, H., Otsuka, M. and Kuroda, H. 1988. Blood pressure lowering activity present in the fruit body of *Grifola frondosa* (Maitake). I. Chem. Pharm. Bull. 36: 1000-1006.
16. Ikekawa, T., Saitoh, H., Feng, W., Zhang, H. L. L. and Matsuzawa, T. 1992. Antitumor activity of *Hypsizigus marmoreus*. I. Antitumor activity of extracts and polysaccharide. Chem. Pharm. Bull. 40: 1954 -1957.
17. Yashioka, Y., Sano, T. and Ikekawa, T. 1973. Studies on antitumor polysaccharide of *Flammulina velutipes (Curt. Ex Fr.) Sing*. I. Chem. Pharm. Bull. 21: 1772-1776.
18. Kubo, K., Aoki, H. and Nanba, H. 1994. Anti-diabetic activity present in the fruit body of *Grifola frondosa* (Maitake). I. Biol. Pharm. Bull. 17: 1106-1110.
19. Kasuga, A., Aoyagi, Y. and Sugahara, T. 1993. Antioxidative activities of several mushroom extracts. Nippon Shokuhin Kogyo Gakkaishi 40: 56-63.
20. Lin, J. M., Lin, C. C., Chen, M. F., Ujiie, T. and Takada, A. 1995. Radical scavenger and antihepatotoxic activity of *Ganoderma formosanum, Ganoderma lucidum* and *Ganoderma neo-japonicum*. J. Ethnopharm 47: 33-41.
21. Kim, K. C. and Kim, I. G. 1999. *Ganoderma lucidum* extract protects DNA from strand breakage caused by hydroxyl radical and UV irradiation. Int. J. Mol. Med. 4: 273-277.
22. Mau, J. L. and Tseng, Y. H. 1998. Nonvolatile taste components of three strains of *Agrocybe cylindracea*. J. Agri. Food Chem. 46: 2071-2074.
23. Kiho, T., Yoshida, I., Nagai, K., Ukai, S. and Hara, C. 1989. $(1 \rightarrow 3)$-α-D-glucan from alkaline extract of *Agrocybe cylindracea*, and antitumor activity of its *O*-(carboxymethyl)ated derivates. Carbohydr. Res. 189: 273-279.
24. Kim, W. G., Lee, I. K., Kim, J. P., Ryoo, I. J., Koshino, H. and Yoo, I. D. 1997. New indole derivative with free radical scavenging activity from *Agrocybe cylindracea*. J. Nat. Prod. 60: 721-723.
25. Lin, S. Y. and Tsai, S. J. 2001. Antioxidant and radical scavenging effects of water extract from *Agrocybe cylindracea*. Bull. Chung Hwa Coll. Med. Technol. 15: 1-10.
26. Shi, X., Dalal, N. S. and Jain, A. C. 1991. Antioxidant behavior of caffeine efficient scavenging of hydroxyl radicals. Food Chem. Toxic. 29: 1-6.
27. Singh, N. P., McCoy, M. T., Tice, R. R. and Schneider, E. L. 1988. A simple technique of quantitation of low levels of DNA damage in individual cells. Exp. Cell Res. 175: 184-191.
28. Ko, J. L., Hsu, C. I., Lin, R. H., Kao, C. L. and Lin, J. Y. 1995. A new fungal immunomodulatory protein *FIP-fve* isolated from the edible mushroom, Flammulina velutipes and its complete amino acid sequence. Eur. J. Biochem. 228: 244-249.
29. Chang, S. K. C. 1994. Protein analysis. In "Introduction to the Chemical Analysis of Foods". Part II Chemical Composition and Characteristics of Foods. p. 213, Nielsen, S. S. ed. Jones and Bartlett Publishers Inc. Boston, London.
30. Low, N. H. 1994. Carbohydrate Analysis. In "Introduction to the Chemical Analysis of Foods". Part II Chemical Composition and Characteristics of Foods. p. 144. Nielsen, S. S. ed. Jones and Bartlett Publishers Inc. Boston, London.
31. Smith, C., Halliwell, B. and Aruoma, O. I. 1992. Protection by albumin against the pro-oxidant actions of phenolic dietary components. Food Chem. Toxic. 30: 483-489.
32. Halliwell, B., Guttteridge, J. M. C. and Aruoma, O. I. 1987. The deoxyribose method: a simple "test tube" assay for determination of rate constants for reactions of hydroxyl radicals. Anal. Biochem. 165: 215-219.
33. Cross, C. E., Halliwell, B., Borisch, E. T., Pryor, W. T., Ames, B. N., Saul, R. L., McCord, J. M. and Harman, D. 1987. Oxygen radicals and human disease. Ann. Intern. Med. 107: 526-545.
34. Halliwell, B., Gutteridge, J. M. C. and Cross, C. E. 1992. Free radicals, antioxidants and human disease; where are we now? J. Lab. Clin. Med. 119: 598-620.
35. Meneghini, R. 1997. Iron homeostasis, oxidative stress, and DNA damage. Free Rad. Biol. Med. 23: 783-792.
36. Aruoma, O. I., Murcia, A., Butler, J. and Halliwell, B. 1993. Evaluation of antioxidant and prooxidant actions of gallic acid and its derivatives. J. Agri. Food Chem. 4:

Journal of Food and Drug Analysis, Vol. 12, No. 3, 2004

1880-1885.
37. Aruoma, O. I. 1994. Deoxyribose assay for hydroxyl radicals. Method. Enzym. 233: 57-66.
38. Liu, F., Ooi, V. E. C. and Chang, S. T. 1997. Free radical scavenging activities of mushroom polysaccharide extracts. Life Sci. 60: 763-771.
39. Sichel, G., Corsaro, C., Scalia, M., DiBilio, A. J. and Bonomo, R. P. 1991. *In vitro* scavenger activity of some flavonoids and melanins against superoxide anions ($O_2^{-}\cdot$). Free Radical Biol. Med. 11: 1-8.
40. Jacobson, E. S., Hove, E. and Emery, H. S. 1995. Antioxidant function of melanin in black fungi. Infect. Imm. 63: 4944-4945.
41. Kim, S. J., Han, D., Park, M. H. and Rhee, J. S. 1994. Screening for superoxide dismutase-like compounds and its activators in extracts of fruits and vegetables. Biosci. Biotech. Biochem. 58: 2263-2265.
42. Pan, S. M. and Ye, J. S. 1997. Screening of SOD activity from edible and medicinal fungi. J. Chin. Agri. Chem. Society 35: 209-219.
43. Liu, M. and Pan, S. M. 1999. Characterization of manganese superoxide dismutase from *Flammulina velutipes*. Food Sci. Agri. Chem. 1: 40-46.
44. Zhu, M., Chang, Q., Wong, L. K., Chong, F. S. and Li, R. C. 1999. Triterpene antioxidants from *Ganoderma lucidum*. Phytotherapy Res. 13: 529-531.
45. Okamura, M. 1994. Distribution of ascorbic acid analogs and associated glycosides in mushrooms. J. Nutr. Sci. Vitaminal. 40: 81-94.