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Antioxidant properties of water-soluble polysaccharides from *Antrodia cinnamomea* in submerged culture

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Abstract

Antrodia cinnamomea, a well-known tradition Chinese medicine, possesses anti-tumor, anti-oxidation activities and stimulates the immune system. The aim of this study was to investigate the protective effect of water-soluble polysaccharides from the fermented filtrate and mycelia of *Antrodia cinnamomea* in submerged culture (ACSC) on hydrogen peroxide-induced cytotoxicity and DNA damage in Chang liver cells. Oxidative DNA damage was evaluated by single cell gel electrophoresis (Comet assay) or by the formation of 8-hydroxy-deoxyguanosine (8-OHdG) adducts. The polysaccharides isolated by ion-exchange chromatography contained glucose, xylose, galactose, arabinose, and mannose. The results showed that incubation of Chang liver cells with isolated polysaccharides at 200 μg/mL for 5 h prior to H_2O_2 treatment (50 μM, 30 min) significantly reduced oxidative DNA damage as detected by the formation of comet tail DNA and 8-OHdG adducts by 89% and 69%, respectively. Pre-treatment Chang liver cells with polysaccharides also reduced the levels of thiobarbituric acid reactive substances (TBARS) ($p < 0.01$) and intracellular reactive species (ROS) ($p < 0.01$) induced by H_2O_2. Moreover, glutathione S-transferase (GST) and the GSH/GSSG ratio were significantly increased in Chang liver cells pre-incubated with the polysaccharides ($p < 0.01$). These results demonstrate that polysaccharides in ASCS have antioxidant properties which may involve up-regulation of GST activity, maintenance of normal GSH/GSSG ratio, and scavenging of ROS.

Keywords: *Antrodia cinnamomea*; Polysaccharide; Antioxidant; DNA damage

1. Introduction

Increasing evidence highlights that oxidative stress-induced cell damage triggers both the physiological process of aging (Harman, 1993) and many pathological progressions that eventually lead to serious health problems such as Parkinson's and Alzheimer's disease (Benzi & Moretti, 1995; Finkel & Holbrook, 2000). Normal energy metabolism in the brain requires that neuronal cells consume a large quantity of oxygen to maintain biochemical processes in body (Kawai, Yonetani, Nakamura, & Okada, 1989). However, reactive oxygen species (ROS) are inevitably generated during normal and/or aberrant consumption of molecular oxygen. These free radicals are able to attack numerous biological substance, including lipid membranes, proteins, and DNA, and exert some detrimental effects, including lipid peroxidation of cell membranes, alteration of lipid-protein interactions, enzyme inactivation, and DNA breakage (Halliwell & Gutteridge, 1998). These oxidized molecules may even result in cell death (Dean, Gieseg, & Davies, 1993). Living cells protect themselves from oxidative damage through several defense mechanisms such as the enzymatic conversion of ROS into less toxic substances (Cotgreave, Moldéus, & Orrenius, 1998) and through detoxification by reaction with antioxidants (Aruoma, 1996).

Antrodia cinnamomea (also known as *Antrodia camphorata*), a *Ganoderma*-like fungus, is a parasite that only lives on *Cinnamonum kanehirai*. *A. cinnamomea* is well known in Taiwan as an expensive medicinal material and is commonly used as an antidote, anticancer, anti-itching and

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hepato-protective drug (Tsai & Liaw, 1985). *A. cinnamomea* is a new species of the genus *Antrodia* (family Polyporaceae, Aphyllophorales) that is parasitic on the inner cavity of the endemic species *Cinnamomum kanehirai* Hay (Chang & Chou, 1995). Although *A. cinnamomea* has a high medicinal value, its fruit body is expensive because of its slow growth rate. Recently, an *A. cinnamomea* submerged culture (ACSC) has been made available. In our previous studies, we demonstrated that ACSC had potent antioxidant activity both *in vitro* and *in vivo* (Song & Yen, 2002, 2003). Hseu et al. (2002) also demonstrated that the protection of normal human erythrocytes against oxidative damage is provided by an aqueous extract of mycelia from ACSC. Song, Hsu, and Yen (2005) also reported that cultured mycelia of ACSC induced apoptosis pathway in hepatoma cells through activation of caspase-8 and -3 cascades and regulation of cell cycle progression. Thus, the cultured mycelia of *Antrodia camphorata* could be a potential agent for chemotherapeutic application.

Among various naturally occurring substances, polysaccharide extracts from mushrooms may prove to be one of the useful candidates in the search for effective, non-toxic substances with free radical scavenging activity (Liu, Ooi, & Chang, 1997; Ooi & Liu, 1999). In our previous study, we found that the polysaccharides in the dry matter filtrate (DMF) and water extract of mycelia (WEM) from ACSC may harbor antioxidant activity (Song & Yen, 2002). However, the antioxidant properties of polysaccharides from ASCS have not been studied. Therefore, the objectives of this study were to investigate whether the polysaccharides in ACSC are capable of reducing H_2O_2-induced DNA damage in Chang liver cells and to elucidate the possible mechanisms.

2. Materials and methods

2.1. Materials

ACSC was obtained from the biotechnology center of Grape King Inc., Chungli, Taiwan. The Chang liver cell line was purchased from Food Industry Research & Development Institute, Hsin Chu, Taiwan. Triton X-100, ethidium bromide, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), glucose standard solution, arabinose, galactose, maltose, xylose, mannose, Amberlite IR-400 and Amberlite IR-120 were purchased from Sigma chemical Co. (St. Louis, MO, USA). Dialysis membrane (Size 27; cut off 12 KD), sodium carbonate (Na_2CO_3), anhydrous sodium bicarbonate ($NaHCO_3$), hydrogen peroxide (H_2O_2), sodium hydroxide (NaOH), sodium chloride (NaCl), barium hydroxide ($Ba(OH)_2$) and barium carbonate ($BaCO_3$) were purchased from Wako Co. (Tokyo, Japan). Dimethyl sulfoxide (DMSO) was obtained from Fluka Chemie (Buchs, Switzerland). The protein assay kit (Bio-Rad Laboratories Ltd, Watford, Herts, U.K.), medium for cell culture (GIBCO BRL, Grand Island, NY) and other reagents or dishes (Nunc, Roskilde, Denmark) were obtained from the indicated suppliers.

2.2. Cells culture

The Chang liver cell line was maintained in Basal medium Eagle powder (BME) (Gibco Co.) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, non-essential amino acid solution (NEAA) and antibiotics (100 U/ml penicillin and 100 μg/mL streptomycin), at 37 °C in a humidified atmosphere of 5% CO_2. The medium was changed every 2 days.

2.3. Culture conditions of *A. cinnamomea*

A. cinnamomea in submerged culture (ACSC) was obtained according to the method of Song and Yen (2002).

2.4. Preparation of polysaccharide extracts from ACSC

The freeze-dried mycelia (about 10 g) from the submerged culture (1 L) were homogenized at 7000 rpm for 2 min at room temperature (homogenizer polytron PT 3000; kinematica Switzerland), then extracted with de-ionized water (2 L) at 30 °C for 24 h, or at 95 °C for 6 h. The extracts were filtered through Whatman No. 2 filter paper, concentrated 6-fold under a vacuum, precipitated with 4 volumes of 95% ethanol, and then allowed to precipitate overnight at 4 °C. The precipitated polysaccharides were collected by centrifugation and lyophilized, resulting in a crude brownish polysaccharide sample named polysaccharide extract of mycelia with cold water (PEMC) and with hot water (PEMH). One liter of mycelial extracellular medium was filtered and concentrated 6-fold under vacuum. The polysaccharide extracts of filtrates (PEF) were obtained in the same way as previous steps.

2.5. Fractionation of polysaccharide extracts

The polysaccharide extracts (0.2 g PEF, 0.15 g PEMH and 0.15 g PEMC) were dissolved in distilled water, dialyzed against distilled water (cutoff MW < 12 KD), and applied to a DEAE-cellulose ion-exchange column. Stepwise elution was performed with distilled water and a linear gradient of NaCl solution (0–2 M). The water-soluble polysaccharides (PEF-1, PEMC-1, and PEMH-1) were collected, lyophilized and stored at 20 °C for further analyses.

2.6. Determination of sugar and protein content and monosaccharide composition of the polysaccharides

Polysaccharide is usually associated with protein as a complex. The contents of polysaccharide and protein in ACSC polysaccharides were determined using the phenol-sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The protein content was determined colorimetrically at 280 nm. 0.1 mg of the polysaccharides was hydrolyzed at 100 °C for 48 h with 5 ml of 2.5 M H_2SO_4 in a sealed test tube. The residue was neutralized with $BaCO_3$ and $Ba(OH)_2$ followed by filtration. The liquid

portion was submitted to an ion exchange chromatography using Amberlite IR-120 (H-form) and Amberlite IRA-400 (Cl-form) resins, and was then neutralized in sugar solution. The sugar solution was dried and resolved in 0.5 mL distilled water for further analysis. An HPLC (Hitachi L-6000 pump & L-5000 LC controller with D-2500 Chromato-Integrator, Hitachi Ltd., Tokyo, Japan) and Sphereclone-CHO column (Phenomenex Inc., Torrance, CA, USA) were used for sugar analysis. The mobile phase was de-ionized water and the column was run at flow rate of 0.4 mL/min. The column temperature was maintained at 90 °C and the injection volume was 20 μL. The detector was a refractive index (RI) detector (Milton Roy, Ivyland, PA, USA).

2.7. Assessment of cell viability

The tetrazolium dye colorimetric test (MTT test) is used to indirectly monitor cell growth indirectly as indicated by the conversion of the tetrazolium salt to the colored product, formazan, the concentration of which can be measured spectrophotometrically (Hansen, Nielsen, & Berg, 1989). Briefly, counted the Chang liver cells were counted with a hemocytometer and seeded in 96-well microplates (2×10^4 cells/well in 200 μL of complete BME medium) for 12 h. Cells were then washed with PBS and incubated with various polysaccharides (0.2 mg/mL) for 24 h. Hundred microliters of medium was removed at the end of the incubation, 10 μL of MTT (5 mg/mL) was added to each well and incubation was allowed to continue for 45 min. Finally, 250 μL of dimethyl sulphoxide (DMSO) was added to each well and the cells were further incubated for 2 h. The plate was read by a FLUOstar galaxy spectrophotometer (BMG Labtechnologies Ltd., Offenburg, Germany) at a wavelength of 570 nm.

2.8. Comet assay of DNA damage

H_2O_2-induced DNA damage was measured using single cell gel electrophoresis (SCGE or Comet assay) (Singh, McCoy, Tice, & Schneider, 1998). Briefly, fully frosted slides were covered with 1% NMA as the first layer, a mixture of cell suspension and 1% LMA as the second layer, and finally with 1% LMA (without cell) as the third layer. After solidification at 4 °C, slides were immersed in the lysing buffer (2.5 M NaCl, 100 mM EDTA, pH 10, with freshly added 1% Triton X-100 and 10% DMSO) at 4 °C for 1 h. The slides were then placed in a horizontal electrophoresis tank (BIO-RAD). The tank was filled with freshly prepared electrophoresis solution (300 mM NaOH, 1 mM EDTA, pH 13, 4 °C), and the slides were left in the solution for 20 min to allow for DNA unwinding and expression of alkali labile damage before electrophoresis. Electrophoresis was then conducted at 4 °C for 20 min at 25 V and 0.3 A. After electrophoresis, the slides were neutralized in neutralizing buffer (0.4 M Tris adjusted to pH 7.5 with hydrochloric acid), stained with ethidium bromide, and kept in a humidified airtight container. They were examined using a fluorescence microscope (Nikon EFD-3) with a 543/10 nm excitation filter BP and a 590 nm emission barrier filter. Images of 100 randomly selected cells from each slide were analyzed with Komet 3.1 (Kinetic Imaging Ltd., Liverpool, UK). One hundred cells on each slide (scored at random) were classified according to the relative intensity of fluorescence in the tail. The degree of DNA damage was scored by determining the percentage of DNA in the tail, $\%\ \text{Tail DNA} = [\text{Tail DNA}/(\text{Head DNA} + \text{Tail DNA})] \times 100$.

2.9. Determination of 8-OHdG in DNA

DNA from Chang liver cells was isolated using the method described by Wei, Cai, and Rahn (1996) with a few modifications. Briefly, Chang liver cells were suspended in 3 mL of nuclei lysis buffer (10 mM Tris–HCl, 100 mM NaCl, 25 mM EDTA, pH 8.2) and following the addition of 0.2 mL of 10% SDS and 0.5 mL of solution containing 1 mg of proteinase K in 0.5% SDS, 2 mM EDTA, they were digested for 5 h in the dark. Proteins was precipitated by adding 1 mL of saturated NaCl followed by centrifugation at 2000*g* at 4 °C for 15 min. Supernatant containing nucleic acid was treated with 2 volumes of cold absolute ethanol in order to precipitate high molecular weight DNA. The precipitate removed with a plastic spatula and was washed with 75% ethanol. After centrifugation, the precipitated DNA was dissolved in 300 μL water. DNA samples were stored at −70 °C until hydrolysis. For hydrolysis of DNA to nucleosides, DNA samples (200 μL) were mixed with 100 μL of 40 mM sodium acetate, 0.1 mM $ZnCl_2$ (pH 5.1), and 20 μL nuclease P1 solution (20 μg protein). Samples were incubated at 37 °C for 1 h. Afterwards, 30 μL of 1 M Tris–HCl (pH 7.4) and 5 μL of alkaline phosphatase solution containing 1.5 units of the enzyme were added to each sample. The reaction mixture was incubated at 37 °C for 1 h. All DNA hydrolysates were ultrafiltered using Ultrafree-MC filter unit (cut off 5000 kD). 8-OHdG and 2′-dG in hydrolysates were determined using HPLC with an electrochemical and UV detector system. DNA hydrolysates were isocratically chromatographed using 20 mM ammonium acetate (pH 5.3)/MeOH (85:15, v/v). Detection of dG was performed at 290 nm. 8-OHdG was detected by the electrochemical detector. The amount of 8-OHdG in DNA was expressed as the number of 8-OHdG molecules per 10^5 dG.

3. Measurement of intracellular ROS

Intracellular ROS was estimated with a fluorescent probe, DCFH-DA (Shen, Shi, & Ong, 1996). DCFH-DA readily diffuses through the cell membrane and is enzymatically hydrolyzed by intracellular esterases to nonfluorescent DCFH, which is then rapidly oxidized to highly fluorescent DCF in the presence of ROS. The DCF fluorescence intensity is believed to parallel the amount of

intracellular ROS. At the end of the incubation, the cells (10^6 cells/mL) were collected and resuspensed with in PBS. An aliquot of the suspension (195 μL) was loaded into a 96-well plate and then 5 ml DCFH-DA was added (final concentration 5 μM). The DCF fluorescence intensity was detected at different time intervals using a FLUO-star galaxy fluorescence plate reader (BMG Labtechnologies GmbH Inc., Offenburg, Germany) with excitation wavelength at 485 nm and emission wavelength at 530 nm.

3.1. Measurement of lipid peroxidation: MDA equivalent levels

Determination of MDA by thiobarbituric acid reactive substance (TBARS) was used as an index of the extent of lipid peroxidation described by Buege and Aust (1978). Cell suspension (1×10^6/mL) were incubated with lotus seed extract at 37 °C for 30 min and centrifuged at 1000 rpm. Then cell pellet was washed twice with PBS and suspended in 0.1 N NaOH 1.4 ml of cell suspension was incubated with 10% TCA and 0.6 M TBA in a boiling water bath for 10 min. Upon cooling, the absorbance was read at 532 nm using a spectrophotometer. The protein content of the cells was determined using a Bio-Rad protein assay kit. TBARS were expressed as malonaldehyde equivalents, calculated using the molar extinction coefficient for MDA, $\varepsilon_{532\ nm} = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$.

3.2. Glutathione S-transferase (GST) activity and GSH/GSSG levels

The GST activity was spectrophotometrically determined according to the method of Habig, Pabst, and Jakoby (1974). GST activity was measured using 0.1 mL of cell lysate, 1 mM 1-chloro-2,4-dinitrobenzene (CDNB), and 1 mM glutathione as substrates in a final volume of 1 mL 0.1 M sodium phosphate buffer (pH 6.5) for 30 min at 37 °C. Enzyme activity was calculated by measuring the change in the absorbance value from the slope of the initial linear portion of the absorbance–time curve at 340 nm for 5 min. The activity is expressed as nmole CDNB-GSH conjugated form/min/mg protein. The protein content of liver homogenates was determined using a Bio-Rad protein assay kit.

GSH/GSSG ratios were determined by the method of Reed et al. (1980). In brief, 100 μL of 10% perchloric acid (PCA) was added to liver homogenates (400 μL) as a precipitant of protein. The mixtures were vortex-mixed and centrifuged (4 °C, 15,000 rpm for 15 min). The pellet was dissolved in 1 N NaOH to analyze protein content. The supernatants (200 μL) were treated with 20 μL of 100 mM iodoacetic acid (IAA), and then neutralized with an excess of sodium bicarbonate. 0.2 mL of 3% 2,4-dinitrofluorobenzene (DNFB) was added to the reaction mixtures which were then allowed to react in the dark at room temperature for 8 h to form the *N*-(2,4-dinitrophenyl) derivatives of *S*-carboxymethyl-GSH and GSSG. The mixtures were centrifuged (15,000 rpm for 15 min) and aliquots of the supernatants were used for the determination of GSH and GSSG by HPLC analysis. The HPLC system consisted of a Hitachi l-6200 pump, a Hitachi D-2500 chromato integrator, a Hitachi L-4200 UV–vis detector, and a 3-aminopropyl column (250 × 4 mm ID, 5 mm particle diameter, Custom Co., Houston, TX). The mobile phase consisted of (A) water-methanol (1: 4, v/v) and (B) 2 M sodium acetate, pH 4.6-methanol (36: 64, v/v). 3-Aminopropyl column was eluted by a 30 min gradient from 75% (A) to 5% and then isocratically maintained for 15 min at a flow rate of 1.2 mL/min with UV detection at 365 nm and a sample injection volume of 20 μL.

3.3. Statistical analysis

All analyses were run in triplicate and averaged. Statistical analyses were performed according to the SAS Institute User's Guide. Analyses of variance were performed using the ANOVA procedure. Significant differences ($P < 0.05$) between the means were determined using Duncan's multiple range tests.

4. Results

4.1. Isolation, fractionation and compositional analysis of polysaccharides

Fig. 1 shows the chromatographic profiles of crude polysaccharides (PEF, PEMC and PEMH) fractionated by DEAE-cellulose column. There were two peaks in the elution profile of PEMC and PEF, whereas the elution profile for PEMH had only one peak. As shown in Fig. 1, PEF-1, PEMC-1 and PEMH-1 were the major water-soluble polysaccharides in polysaccharide extracts of ACSC. PEMC-1 and PEMH-1 exhibited absorbance at 280 nm, indicating that mycelial polysaccharides were protein-containing glycan. In contrast, PEF-1, which showed no significant absorbance at 280 nm, was free of any peptide chain.

The monosaccharide composition of various polysaccharides in ACSC is shown in Table 1. Glucose was the most abundant monosaccharide in polysaccharides of ACSC, and mannose was the second most abundant. PEMC-1 and PEF-1 had about 1.15% and 0.03% of xylose, respectively, and PEMC-1 also had 4.17% of arabinose.

4.2. Effects of polysaccharides on cytotoxicity, % tail DNA and 8-OHdG content in Chang liver cells

Table 2 shows that cellular viabilities were all higher than 90% when Chang liver cells were treated with various polysaccharides from ACSC at concentration up to 200 μg/mL at 37 °C for 24 h, indicating that the polysaccharides from ASCS were not cytotoxic to Chang liver cells. Consistent with the results of the MTT assay, cells treated with 200 μg/mL of various polysaccharides at the concentration of the 200 μg/mL for 24 h did not show any obvious

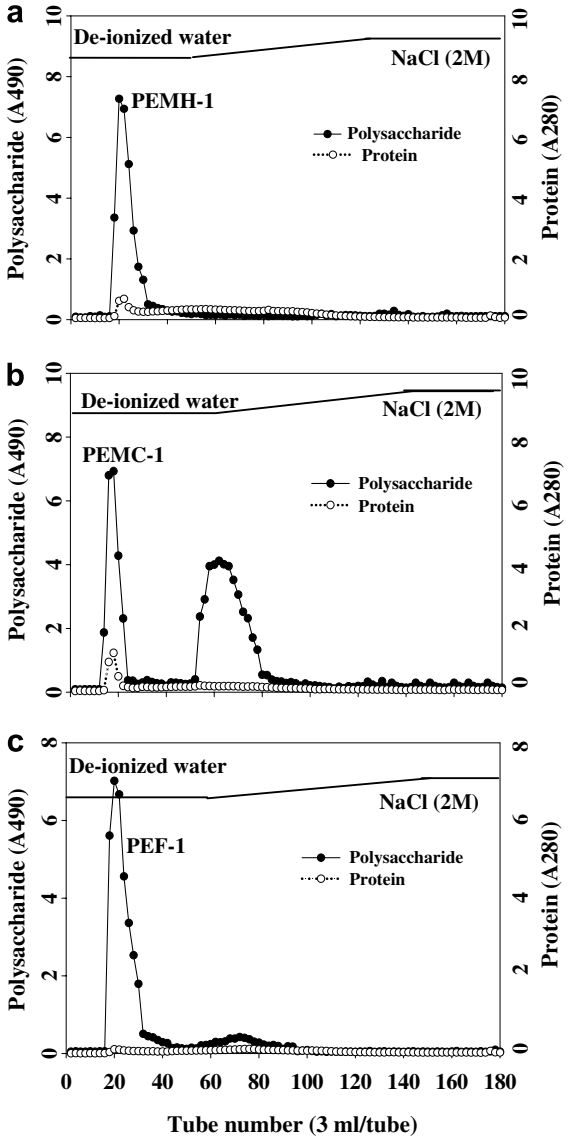

Fig. 1. Fractionation of the isolated polysaccharides (PEMH-1, PEMC-1, PEF-1) in ACSC with a DEAE-cellulose ion-exchange column. Extract was loaded onto the DEAE column (2.8 × 40 cm), de-ionized water was used to elute the unbound component (PEMH-1 (a), PEMC-1 (b); PEF-1 (c)), and the retained components were eluted with an NaCl salt gradient (0-2 M), at a flow rate of 24 mL/h.

Table 1
Monosaccharide composition of polysaccharides in ACSC

Polysaccharides[a]	Composition of polysaccharide (%)				
	Glucose	Xylose	Galactose	Arabinose	Mannose
PEF-1	73.65	0.027	–[b]	–	5.22
PEMC-1	69.42	1.150	–	4.17	8.67
PEMH-1	47.74	–	–	–	15.65

[a] Polysaccharide extracts were fractionated with a DEAE-cellulose ion-exchange column into filtrate-1 (PEF-1), mycelia with cold water-1 (PEMC-1) and mycelia with hot water-1 (PEMH-1).
[b] Not detectable.

increase in % tail DNA and 8-OHdG content. Therefore, 200 μg/mL of polysaccharides from ACSC was used for the following cell culture experiment.

Table 2
Effect of polysaccharides from *A. cinnamomea* in submerged culture (ACSC) on cytotoxicity, % tail DNA and 8-OHdG content in Chang liver cells[a]

Polysaccharides[b] (200 μg/ml)	Cell viability (%)	Tail DNA (%)	8-OHdG/10^5 dG
Control	100 ± 2	5.8 ± 1.0	0.5 ± 0.2
PEMH-1	93 ± 10	5.7 ± 0.8	0.4 ± 0.2
PEMC-1	101 ± 2	5.3 ± .06	0.3 ± 0.2
PEF-1	106 ± 10	5.0 ± 0.4	0.4 ± 0.2

[a] Chang liver cells were incubated with 200 μg/mL of polysaccharides for 24 h.
[b] Polysaccharide extracts were fractionated with a DEAE-cellulose ion-exchange column into filtrate-1 (PEF-1), mycelia with cold water-1 (PEMC-1) and mycelia with hot water-1 (PEMH-1).

4.3. Effect of polysaccharides on H_2O_2-induced DNA damage

Fig. 2 shows the effects of pre-treatment of Chang liver cells with polysaccharides (200 μg/mL) (PEMH-1, PEMC-1, PEF-1) from ACSC on DNA damage induced by H_2O_2 (50 μM). The results showed that H_2O_2 induced 43% tail DNA, which was reduced to about 8% in cells pretreated with each polysaccharide. Similarly, 8-OHdG adduct formation was significantly inhibited (by 69%, $p < 0.01$) in Chang liver cells pretreated with 200 μg/ml of PEMH-1, PEMC-1 and PEF-1. However, no significant differences in the ability of PEMH-1, PEMC-1 and PEF-1 to inhibit either % tail DNA or 8-OHdG were observed.

4.4. Effects of polysaccharides on intracellular ROS and TBARS formation in H_2O_2-treated cells

Pretreatment of cells with various polysaccharides at the concentration of 200 rmug/mL significantly decreased the formation of ROS ($p < 0.01$) induced by H_2O_2 (Fig. 3). Among the three fractions of polysaccharides, PEMH-1 was significantly more effective than PEMC-1 and PEF-1 at inhibiting formation of intracellular ROS (68% decrease by PEMH-1 and 50–58% decrease by PEMC-1 and PEF-1). Fig. 4 shows that pretreatment of cells with PEMH-1, PEMC-1 or PEF-1 (200 μg/ml) significantly decreased the level of TBARS induced by H_2O_2 ($p < 0.01$). However, no significant differences in the ability of PEMH-1, PEMC-1 and PEF-1 to inhibit TBARS formation ($p > 0.01$) were observed.

4.5. Effects of polysaccharides on GST activity and the GSH/GSSG ratio in cells

Table 3 shows the effect of PEMH-1, PEMC-1 and PEF-1 on GST activity and on the GSH/GSSG ratio in H_2O_2-treated Chang liver cells. The GST activity and the GSH/GSSG ratio in Chang liver cells were decreased 33% and 30%, respectively, after treatment with H_2O_2. Pretreatment of cells with 200 μg/ml of polysaccharides from ACSC at the concentration of 200 μg/ml for 5 h significantly

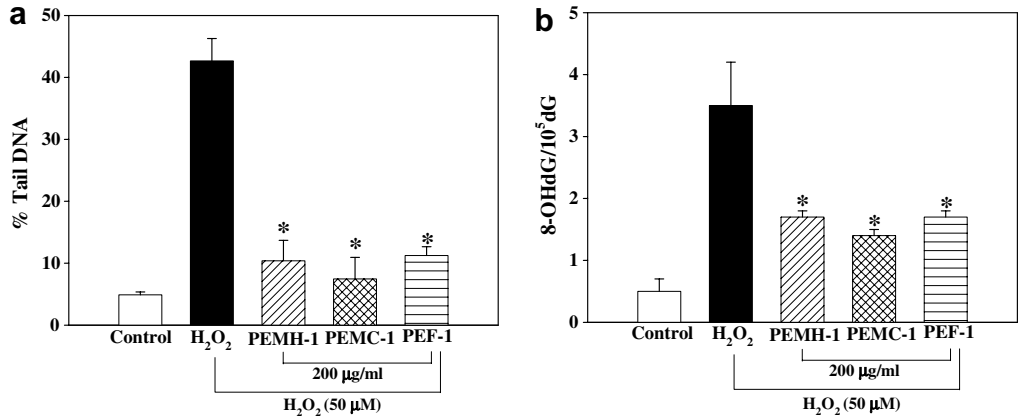

Fig. 2. Protective effect of polysaccharides from ACSC against H_2O_2-induced oxidative DNA damage in Chang liver cells. DNA damage in cells was estimated with the comet assay (a) and 8-OHdG adducts (b). Polysaccharide extracts were fractionated with a DEAE-cellulose ion-exchange column into filtrate-1 (PEF-1), mycelia with cold water-1 (PEMC-1) and mycelia with hot water-1 (PEMH-1). The cells were treated with polysaccharides (200 μg/mL) for 5 h prior to H_2O_2 (50 μM) treatment (30 min). The oxidized DNA bases are expressed as the number of 8-OHdG per 10^5 dG. Control indicates that cells were treated with PBS only. Data are presented as mean ± SD ($n = 3$). $^*p < 0.01$ compared to the group treated with H_2O_2 only.

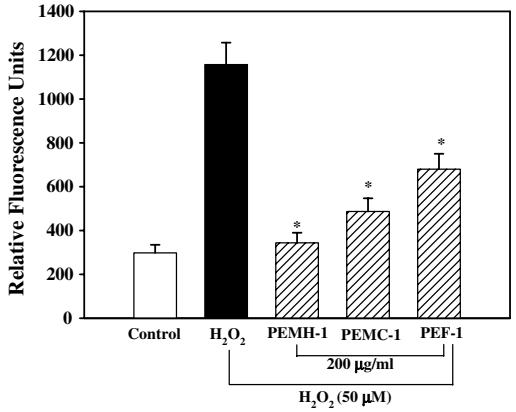

Fig. 3. Effect of polysaccharides (200 μg/mL) from ACSC on intracellular ROS level in H_2O_2-treated Chang liver cells. Polysaccharide extracts were fractionated with a DEAE-cellulose ion-exchange column into filtrate-1 (PEF-1), mycelia with cold water-1 (PEMC-1) and mycelia with hot water-1 (PEMH-1). The cells were pre-incubated with polysaccharides for 5 h prior to H_2O_2 (50 μM) treatment (30 min). Control indicates that cells were treated with PBS only. Data were presented as mean ± SD ($N = 3$). $^*p < 0.01$ compared to the group treated with H_2O_2 only.

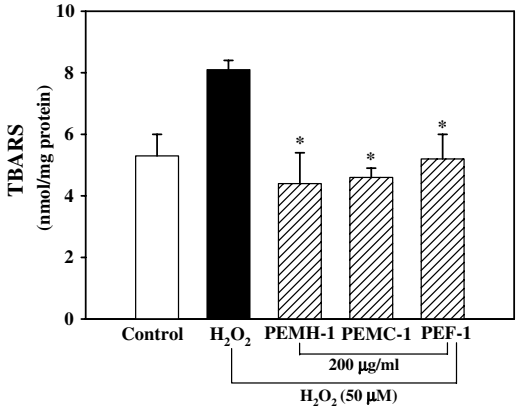

Fig. 4. Effect of polysaccharides (200 μg/mL) from ACSC on H_2O_2-induced lipid peroxidation products formation in Chang liver cells. Polysaccharide extracts were fractionated with a DEAE-cellulose ion-exchange DEAE-cellulose into filtrate-1 (PEF-1), mycelia with cold water-1 (PEMC-1) and mycelia with hot water-1 (PEMH-1). The cells were pre-incubated with polysaccharides for 5 h prior to H_2O_2 (50 μM) treatment (30 min). Control indicates that cells were treated with PBS only. Data were presented as mean ± SD ($N = 3$). Values are significantly different by comparison with the control (H_2O_2 alone), $^*p < 0.01$.

increased GST activity and the GSH/GSSG ratio which were decreased by H_2O_2treatment ($p < 0.05$). Neither GST activity nor the GSH/GSSG ratio was significantly different among PEMH-1, PEMC-1 and PEF-1 treatments ($p > 0.05$).

5. Discussion

Among various naturally occurring substances, polysaccharides from mushrooms may prove to be one of the useful candidates in the search for effective, non-toxic substances with antioxidant activity (Li et al., 2003; Zhou, Chen, Ouyang, Liu, & Pang, 2000). The multifunctions of polysaccharides have been extensively investigated. Wu, Chen, and Xie (2006) reported that polysaccharide from

Table 3
Effects of polysaccharides on GST activity and GSH/GSSG ratio in H_2O_2-treated Chang liver cells[a]

Polysaccharides (200 μg/mL)	GST activity (nmol/min/mg protein)	GSH/GSSG ratio
Control	18 ± 1^a	13.1 ± 0.2^c
H_2O_2	12 ± 3^b	9.2 ± 0.6^d
PEF-1	17 ± 2^a	17.2 ± 0.5^a
PEMC-1	19 ± 1^a	14.6 ± 0.9^b
PEMH-1	20 ± 2^a	14.0 ± 0.2^b

[a] Chang liver cells were pre-incubated with polysaccharides for 5 h before exposure to H_2O_2 for 30 min. Control indicates that cells were treated with PBS only. Chang liver cells were pre-incubated with polysaccharides for 5 h prior to H_2O_2 (50 μM) treatment (30 min). Values in column with the different superscripts are significantly different ($p < 0.05$). Results are mean ± SD for $n = 3$.

Gekko swinhonis Guenther, which has been used as traditional herbal medicine in China for hundreds of years, shows apoptosis-inducing effects on hepatocarcinoma cells. Moreover, Chen, Yan, Zhu, and Lin (2006) have showed that red seaweed polysaccharides can exert antioxidant capacity against carbon tetrachloride-induced heptatotoxicity. Although the exactly molecular mechanisms of hepatoprotection effects of polysaccharides have not been revealed yet, both *in vitro* and *in vivo* studies all show that polysaccharides from natural occurring ingredients with various bioactivities. Results from our previous study suggested that the polysaccharides might possess antioxidant activity in the dry matter filtrate (DMF) and water extract of mycelia (WEM) of *A. cinnamomea* (Song & Yen, 2002). Here, we took human liver normal hepatocyte as model cells to examine whether polysaccharides in ACSC are capable of reducing the H_2O_2-induced oxidative damage to mimic the oxidative stress-mediated destruction in liver. DMF and WEM were precipitated with ethanol to enrich crude polysaccharides with a cutoff of 12 kD. The polysaccharide extracts were further fractionated by ion-exchange chromatography into filtrate-1 (PEF-1), mycelia with cold water-1 (PEMC-1) and mycelia with hot water-1 (PEMH-1) to determine which fraction was reducing oxidative damage. H_2O_2 was used in the present study to induce oxidative injury in Chang liver cells because H_2O_2 is known to increase in pathological processes, which leads to oxidative DNA damage (Sies, 1985).

Our results revealed that water-soluble polysaccharides from ACSC exhibited significant and dose-dependent protection (up to 200 μg/mL) against H_2O_2-induced DNA damage as evidenced by the decreased levels of % tail DNA and 8-OHdG. Kim, Kacew, and Lee (1999) also found that the polysaccharides from *Ganoderma lucidum*, *Coriolus versicolor* and *Lentinus edodes* could protect DNA from damage by using benzo[*a*]pryene to induce 8-OHdG in NCTC-clone 1469 cells. We explored the mechanism underlying the protective effect of polysaccharides in ACSC on H_2O_2-induced oxidative damage in Chang liver cells. The results indictaed that polysaccharides from ACSC were capable of suppressing intracellular ROS and TBARS, a product of lipid peroxidation, in Chang liver cells exposed to 50 μM H_2O_2 for 30 min.

We also determined the activity of GST in Chang liver cells because polysaccharides from other mushrooms have been reported to inhibit oxidative damage in cells treated with carcinogens by increasing the activity of GST (Bilzer et al., 1984; Kim et al., 1999). In the present study, the results showed that the polysaccharides from ACSC elevated GST activities that were decreased by H_2O_2. Similarly, the results also showed that pre-treatment of Chang liver cells with the polysaccharides significantly raised the GSH/GSSG ratios ($p < 0.01$) that were decreased by H_2O_2. These results demonstrate that polysaccharides from ACSC have antioxidant properties *in vitro* and that the possible mechanisms of the polysaccharides may involve up-regulation of GST activity, maintenance of a normal GSH/GSSG ratio, and scavenging of ROS. The somewhat different monosaccharide compositions in PEF-1, PEMC-1 and PEMH-1 did not appear to influence the antioxidant activity of these polysaccharide fractions, since these fractions exhibited no significant differences in their ability to inhibit lipid peroxidantion, % tail DNA or 8-OHdG adduct formation ($p > 0.05$). It is currently unclear how polysaccharides affect the intracellular antioxidant system. The polysaccharides from ASCS may contain biological information since these glycoproteins contain several types of essential sugars (e.g. glucose, mannose, xylose) that predominate in human glycoproteins and glycoprotein receptors (Murray, 2003).

In conclusion, the present study demonstrates that water-soluble polysaccharides from ACSC are capable of protecting against H_2O_2-induced DNA damage in Chang liver cells. The results suggest that the water-soluble polysaccharides may contribute to the medicinal functions of ACSC.

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