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Antrodia salmonea in submerged culture exhibits antioxidant activities *in vitro* and protects human erythrocytes and low-density lipoproteins from oxidative modification

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ABSTRACT

Antrodia salmonea is well known in Taiwan as a beneficial mushroom. In the present study, we investigated the antioxidant activity of whole fermented broth (AS), filtrate (ASF), and mycelia (ASM) of *A. salmonea* using different antioxidant models. Furthermore, the effect of *A. salmonea* on AAPH-induced oxidative hemolysis of human erythrocytes and $CuSO_4$-induced oxidative modification of human low-density lipoproteins (LDLs) was examined. We found that the AS, ASF, and ASM possess effective antioxidant activity against various oxidative systems including superoxide anion scavenging, reducing power, metal chelation, and DPPH radical scavenging. Further, AAPH-induced oxidative hemolysis in erythrocytes was prevented by AS, ASF, and ASM. Notably, AS, ASF, and ASM appear to possess powerful antioxidant activities against $CuSO_4$-induced oxidative modification of LDL as assessed by malondialdehyde (MDA) formation, cholesterol degradation, and the relative electrophoretic mobility of oxidized LDL. It is noteworthy that AS had comparatively strong antioxidant ability compared to ASF or ASM, which is well correlated with the content of their total polyphenols. Thus, *A. salmonea* may exert antioxidant properties and offer protection from atherogenesis.

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1. Introduction

In biological systems, free radicals can be generated in the form of reactive oxygen species (ROS) such as hydrogen peroxide, nitric oxide, superoxide anion, hydroxyl radicals, singlet oxygen, and peroxynitrites (Trachootham et al., 2008). These ROS can cause irreversible damage to cellular components including lipids, proteins, and DNA (Trachootham et al., 2008). Erythrocytes, potentially powerful promoters of oxidative processes, are extremely susceptible to oxidative damage because of the high polyunsaturated fatty acid content of their membranes and their high cellular oxygen and hemoglobin concentrations (Pandey and Rizvi, 2010). A number of reports have shown that the attack of erythrocytes by ROS is a key event in β-thalassemia, sickle cell anemia, glucose-6-phosphate dehydrogenase deficiency, and other hemoglobinopathies (Vellosa et al., 2011). Malondialdehyde (MDA), a well-characterized product of erythrocyte lipid peroxidation, is a highly reactive and bifunctional molecule that has been shown to cross-link erythrocyte phospholipids and proteins. This crosslinking impairs a variety of membrane-related functions and ultimately diminishes erythrocyte survival (hemolysis) (Cimen, 2008). Furthermore, erythrocyte lipid peroxidation and hemolysis may be involved in a variety of pathological events in normal cell aging (Vellosa et al., 2011).

Increasing evidence suggests that oxidation of low-density lipoprotein (LDL) plays a major role in the development of atherosclerosis (Niki, 2011). Oxidized LDL is considered a major risk factor for

Abbreviations: ROS, reactive oxygen species; $CuSO_4$, copper sulfate; AAPH, 2,2′-azo-bis(2-amidinopropane) hydrochloride; DPPH, 1,1-diphenyl-2-picryl-hydrazil; LDL, low-density lipoproteins; TBARS, thiobarbituric acid-reactive substances; MDA, malondialdehyde.

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atherosclerosis. Oxidized LDL is taken up by arterial wall macrophages at an enhanced rate, leading to cellular cholesterol accumulation and foam cell formation (Li and Glass, 2002). The atherogenicity of oxidized LDL includes cytotoxicity against arterial wall cells, accompanied by pro-inflammatory and thrombotic effects (Levitan et al., 2010). Supplementation with antioxidants is known to increase LDL resistance to oxidation both *in vitro* and *in vivo* (McEneny et al., 2007). Furthermore, the inhibition of LDL oxidation can retard the onset of atherosclerosis in animal models (Stocker and O'Halloran, 2004). The increased intake of antioxidants is also associated with a decreased incidence of new or recurrent cardiovascular disease (Myung et al., 2013) as well as reduced angiographic progression (Laight et al., 2000). Therefore, it seems reasonable to suggest that the antioxidants in foods and/or drugs may play a role in the prevention of free radical-related diseases and atherosclerosis.

Antrodia salmonea (or *Taiwanofungus salmoneus*), a newly identified medicinal fungal species, belongs to the genus *Taiwanofungus*. The fruiting body of *A. salmonea* has been used in Taiwanese folk medicine for the treatment of diarrhea, abdominal pain, hypertension, itchy skin, and liver cancer. It has also been used as a food and drug detoxicant (Shen et al., 2008). However, very few biological activity tests of this fungus have been reported. To date, several new compounds have been isolated from the basidiomata of *A. salmonea*, and *in vitro* studies of these compounds demonstrated their anti-oxidative (Shen et al., 2006) and anti-inflammatory activities in activated macrophage cells (Huang et al., 2012). Recently, the effects of various phytochemicals on health, especially the suppression of reactive oxygen species by natural antioxidants from teas, spices, and herbs, have been studied intensively (Reddy et al., 2010). The aim of the present study was to examine the antioxidant effects of the fermented culture broth (AS), filtrate (ASF), and mycelia (ASM) of *A. salmonea*, including its reducing power and its superoxide anion scavenging, metal chelating, and DPPH radical scavenging activities. AS was also used to inhibit the 2,2′-azo-bis(2-amidinopropane) hydrochloride (AAPH)-induced oxidative hemolysis of human erythrocytes. Furthermore, we investigated the effects of AS on the oxidative modification of LDL induced by $CuSO_4$, as determined using MDA formation, cholesterol degradation, and electrophoretic mobility assays.

2. Materials and methods

2.1. Chemicals

Sodium citrate, sodium chloride (NaCl), sodium phosphate dibasic (anhydrous) (Na_2HSO_4), bovine serum albumin (BSA), copper sulfate ($CuSO_4$), 1,1-diphenyl-2-picryl-hydrazil (DPPH), 2-thiobarbituric acid (TBA), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), ethylenediaminetetraacetic acid (EDTA), vitamin C, gallic acid, and vitamin E (Trolox) were obtained from Sigma–Aldrich (St. Louis, MO). AAPH, phosphoric acid (H_3PO_4), and trichloroacetic acid were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). All other chemicals were of reagent grade and were supplied by either Merck (Darmstadt, Germany) or Sigma–Aldrich.

2.2. A. salmonea from submerged cultures

The *A. salmonea* hyphae were separated from the fruiting bodies. The whole colony was cut and placed into a flask with 50 mL of sterile water. After homogenization, the fragmented mycelia suspension was inoculated with a culture medium composed of 2.0% glucose, 0.1% wheat powder, and 0.1% peptone in distilled water. The medium was adjusted to an initial pH of 5.0. Each culture was grown in a 2 L Erlenmeyer flasks (containing 1 L of medium) and incubated with shaking at 120 rpm at 25 °C for 10 days. Then, 3.5 L of the shaking flask cultures were inoculated into a 500 L fermenting tank containing 300 L of culture medium and cultured at 25 °C for 30 days. The fermentation conditions were the same as those used for the seed fermentation but with an aeration rate of 0.075 vvm to produce a mucilaginous medium containing the mycelia. The experiments were performed using 2–4 different batches of the whole fermented culture of *A. salmonea*.

2.3. Sample preparation

The fermentation product was poured through a non-woven fabric on a 20 mesh sieve to separate the deep yellow fermented culture into the filtrate and mycelia and then concentrated under a vacuum and freeze dried. The dry matter yield of the fermented culture, filtrate, and mycelia were approximately 15, 5, and 10 g/L, respectively. The freeze-dried samples were ground, shaken with distilled water, and then centrifuged at 3000×*g* for 5 min, followed by passage through a 0.2 μm filter. The aqueous extracts were concentrated in a vacuum and freeze dried to form a powder. The yields of the fermented culture broth (AS), filtrate (ASF), and mycelia (ASM) (1 g) were approximately 0.375, 0.75, and 0.075 g, respectively. To prepare the stock solution, the powdered samples of AS, ASF, and ASM were solubilized with 10 mM sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl (PBS) at 25 °C. The solution was stored at −20 °C prior to the analysis of the antioxidant properties.

2.4. HPLC separation and peak identification

The HPLC profile of the MeOH extracts of fermented culture broth from *A. salmonea* (AS) was determined using a RP-18 column [COSMOSIL, $5C_{18}$-AR-II, Waters, 4.6 × 250 mm, 5 μm] at a flow rate of 1.0 mL/min and detection at a UV wavelength of 280 nm. A standard solution of AS was prepared by dissolving the AS in MeOH (10 mg/mL), and the solution was then filtered through a 0.22 μm membrane filter and applied to the HPLC for analysis. The mobile phase consisted of (A) MeOH and (B) 0.1% trifluoroacetic acid (TFA) (v/v), and a gradient elution of 20–95% A at 0–30 min and 95–100% A at 30–40 min was performed. The flow rate was maintained at 1.0 mL/min. Aliquots of 20 μL were injected applied to the HPLC for analysis. We further characterized the main composition of AS using chromatography followed by spectral analysis. Both 1D and 2D NMR spectra were determined on a Bruker NMR spectrometer (Unity Plus 400 MHz) using C_5D_5N as solvent for measurement. All separations were performed using a Shimadzu LC solution program. The system included a pump (LC-20AT), an autosampler (SIL-20), a column oven (CTO-20A), and a PDA detector (SPD-M20A). LC–MS was performed using the Agilent 1100 series, a mass Esquire HCT (Bruker), and an Agilent Zorbax Eclipse SB-C18 column (2.1 × 150 mm, 5 μm).

2.5. Superoxide anion scavenging activity

The superoxide anion scavenging activity of AS, ASF, and ASM was measured using the method described by Robak and Gryglewski, with slight modifications (Robak and Gryglewski, 1988). Superoxide radicals were generated in phenazine methosulphate (PMS)-nicotinamide adenine dinucleotide (NADH) systems by NADH oxidation and assayed by nitroblue tetrazolium (NBT) reduction. In this experiment, the generated superoxide radicals were incubated with various concentrations of AS, ASF, or ASM (30–200 μg/mL). The reaction was initiated by adding 0.5 mL of the PMS solution (120 μM) to the mixtures. The reaction mixture was incubated at 25 °C for 5 min, and the absorbance at 560 nm was measured against blank samples using a spectrophotometer. Vitamin C and E were used as positive controls for the antioxidant activity tests.

2.6. Reducing power

The reducing powers of AS, ASF, and ASM were determined using the method proposed by Oyaizu (1986). Various concentrations of AS, ASM, and ASF (30–200 μg/mL) were mixed with phosphate buffer (pH 6.6) and potassium ferric cyanide [$K_3Fe(CN)_6$] and incubated at 50 °C for 20 min. Then, trichloroacetic acid was added, and the mixture was centrifuged for 10 min at 1000×*g*. The upper layer of the solution (0.5 mL) was mixed with distilled water (0.5 mL) and $FeCl_3$ (0.1 mL) for 10 min, and the absorbance was then measured at 700 nm in a spectrophotometer. Higher absorbance values indicate greater reducing power.

2.7. Metal chelating activity

The chelation of ferrous ions by AS, ASF, and ASM was estimated using the method described by Dinis et al. (1994). Briefly, 0.94 mL of various concentrations of AS, ASF, and ASM (30–200 μg/mL) was added to a solution that contained 0.02 mL of $FeCl_2$ (2 mM). The reaction was initiated by the addition of 0.04 mL of ferrozine (5 mM), after which the mixture was shaken vigorously and allowed to stand at room temperature for 10 min. After equilibrium had been reached, the absorbance of the solution was measured at 562 nm using a spectrophotometer.

2.8. DPPH free radical scavenging activity

The free radical scavenging activities (hydrogen donation) of AS, ASF, and ASM were measured using the DPPH assay (Yokozawa et al., 1998). Briefly, a solution of DPPH in methanol (200 μM) was prepared. Then, 1 mL of DPPH solution was added to 0.4 mL of various concentrations of ASB, ASF, and ASM (30–200 μg/mL). The

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