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Novel Antioxidant Peptides from Fermented Mushroom *Ganoderma lucidum*

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Oxidative stress has been linked with the pathogenesis of many human diseases including cancer, aging, and atherosclerosis. The present study investigates the antioxidant activities of peptides isolated from the medicinal mushroom, *Ganoderma lucidum*. *G. lucidum* has been shown to possess potent antioxidant activity with little or no side effects. Polysaccharide, polysaccharide−peptide complex, and phenolic components of *G. lucidum* have been proposed to be responsible for this antioxidant effect. However, research has shown that the *G. lucidum* peptide (GLP) is the major antioxidant component of *G. lucidum*. The objective of this study was to evaluate the antioxidant activity of this peptide using different oxidation systems. GLP showed potent antioxidant activities in both lightproof soybean oil and lard systems, assessed by lipid peroxidant value. Compared to butylated hydroxytoluene, GLP showed a higher antioxidant activity in the soybean oil system. Soybean lipoxygenase activity was blocked by GLP in a dose-dependent manner with an IC_{50} value of 27.1 μg/mL. GLP showed scavenging activity toward hydroxyl radicals produced in a deoxyribose system with an IC_{50} value of 25 μg/mL, and GLP effectively quenched superoxide radical anion produced by pyrogallol autoxidation in a dose-dependent manner. Malondialdehyde level has been used as the oxidation index in many biological systems. GLP showed substantial antioxidant activity in the rat liver tissue homogenates and mitochondrial membrane peroxidation systems. The auto-hemolysis of rat red blood cells was also blocked by GLP in a dose-dependent manner. On the basis of these results, it is concluded that GLP is the major constituent responsible for the antioxidant activity of *G. lucidum*. GLP could play an important role in the inhibition of lipid peroxidation in biological systems through its antioxidant, metal chelating, and free radical scavenging activities.

KEYWORDS: Peptide; medicinal mushroom; *Ganoderma lucidum*; antioxidant activity; free radical scavenging activity; antioxidant components

INTRODUCTION

Recent research has established the role of reactive oxygen species (ROS) in the pathogenesis of certain human illnesses including cancer, aging, and atherosclerosis (*2*, *3*). Oxidation of biomolecules, including lipid peroxidation, involves a series of free radical mediated chain reactions and is associated with several types of biological damage. Therefore, much attention has been focused on the use of antioxidants, especially natural antioxidants, to inhibit lipid peroxidation and to protect biomolecules from damage by free radicals (*4*).

Ganoderma lucidum, known as Ling Zhi in China, has been shown to possess potent antioxidant activity in multiple research studies with little or no side effects (*1*, *5*). In a human intervention study, ingestion of *G. lucidum* supplement was found to cause an acute increase in plasma antioxidant capacity (*6*). *G. lucidum* polysaccharides have been proven to be the bioactive constituents responsible for many health benefits of *G. lucidum*, such as anticancer and immune-stimulatory effects (*7*). However, the active components responsible for the antioxidant activity of *G. lucidum* are still not clear. *G. lucidum* and its polysaccharide, polysaccharide−protein, and phenolic constituents were reported to possess antioxidant activity, to inhibit lipid peroxidation, and to scavenge free radicals (*1*, *5*, *8*−*10*). Recently, Chung et al. demonstrated that *G. lucidum* could react with the cell membrane and lower lipid peroxidation in situ (*11*). Moreover, in a previous study, we found that bioactive peptides in the water-soluble fraction of *G. lucidum* may be one of the contributors to the total antioxidant activity of *G. lucidum* (*12*).

In many plant and animal materials, the bioactive peptides (BAP), defined as peptides with molecular masses of <6000 Da, have been found to possess antioxidant properties (*13*). An important feature of BAP is that the human intestine easily absorbs these peptides, especially di- and tripeptides (*14*, *15*). Using the rat as a model, Hara et al. showed that absorption of small peptides was 70−80% higher than that of free amino acids (*16*). In another experiment involving duodenal infusion of samples into pigs, milk enzymatic hydrolysate showed better

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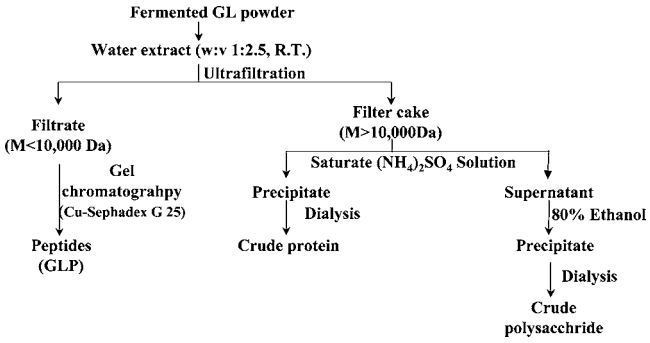

Figure 1. Flowchart of peptide separation from fermented *G. lucidum* powder.

amino acid absorption than free amino acids (*17*). An immunomodulatory protein, Ling Zhi-8 (LZ-8), has been isolated and successfully synthesized in the mycelia of *G. lucidum*, with a molecular mass range from 13100 to 17500 Da, depending on the testing system employed (*18*, *19*). The major bioactivity of LZ-8 lies in its immunomodulatory capacity. However, no antioxidant activity of LZ-8 has ever been reported. Some of the BAPs from other plant species, on the other hand, have been shown to be potent antioxidants (*20*, *21*). Turmerin, a water-soluble peptide from turmeric, for example, has been shown to afford strong protection to membranes and DNA against oxidative injury (*20*). Additionally, Kumar et al. showed that a tetrapeptide derivative [Boc-Lys-(Boc)-Arg-Asp-Ser-(tBu)-OtBu], corresponding to residues 39−42 of human lactoferrin, exhibited a significant antiarthritic activity by increasing the antioxidant levels in rats (*21*). For the first time, we have successfully separated *G. lucidum* peptides (GLP) and have shown that the GLP has more potent hydroxyl free radical scavenging activity than protein and polysaccharide fractions of *G. lucidum* (*12*, *22*).

This study aimed to evaluate the antioxidant properties of GLP in food lipid and animal biological systems and to evaluate the free radical scavenging activities of GLP on different biological free radicals.

MATERIALS AND METHODS

Chemicals and Reagents. Thiobarbituric acid (TBA) and 2-deoxy-D-ribose (DR) were purchased from Sigma Chemical Co. (St. Louis, MO). Malondialdehyde (MDA) detection kit was purchased from JianChen Biological Engineering Institute (Nanjin, China). Lipoxygenase was extracted from fresh soybean and purified in our laboratory. All other chemicals used were of analytical purity. Soybean oil was purchased at a local market (Wuhan, China).

***G. lucidum* Samples and GLP Preparation (*22*).** Fermented *G. lucidum* was purchased from Guoyao Co. (Wuhan, China). The peptides were separated from fermented *G. lucidum* as shown in the flowchart (**Figure 1**). Briefly, *G. lucidum* water extraction (GLW) was separated into 70% filtrate and 30% retentate by an Ultrafiltrate system with a 10000 Da membrane (*12*). The peptide was further purified from the filtrate by using a gel-based ligand chromatogram method specifically designed for low molecular weight peptide purification (*23*). The Blue-Sephadex G-25 complex is first prepared by slowly adding Sephadex G-25 to 0.16 mol/L $CuSO_4$ (0.53 g/mL) followed by adding 0.5 mol/L NaOH ($2 \times V_{CuSO_4}$) with constant stirring. Then the final product, Cu-Sephadex G-25, was formed by adding tetraborate solution (pH 11.0, 50 mmol/L, $2 \times V_{CuSO_4}$) and allowing the blue solid to sink gradually (*23*). The Cu-Sephadex G-25 gel then was packed into a glass column (∅ 1 cm × 45 cm). One gram of freeze-dried filtrate was reconstituted in 1 mL of dH_2O, and borate buffer (pH 11.0, 50 mmol/L) was used as the elution buffer with an elution speed of 1.2 mL/min. Peptide fraction was detected at UV-254 nm and collected by an autocollector with a speed of 5 mL/tube. The copper was removed from the Cu-peptide chelates by means of sodium diethyldithiocarbamate (*23*). The purity of the peptide fraction was 91.5% determined by amino acids analysis. The water extracts (GLW, dry weight) composed about one-third of the raw material dry weight. The isolated peptides accounted for ∼5.2% of the raw material and 13.9% of the GLW.

DR Assay for Hydroxyl Radical. The DR assay was used to evaluate the hydroxyl radial scavenging ability of GLP (*24*). Briefly, the following reagents were added into a reaction tube in order: 0.4 mL of KH_2PO_4−KOH buffer (pH 7.5), 0.1 mL sample solution of various concentrations, and 0.1 mL each of 1 mM EDTA, 10 mM H_2O_2, 60 mM DR, 2 mM ascorbic acid, and 1 mM $FeCl_3$. Solutions of $FeCl_3$, H_2O_2, and ascorbic acid were made just before use. The reaction solution was incubated at 37 °C for 1 h. Then 1 mL of 25% (v/v) HCl was added to stop the reaction. The color was developed by adding 1 mL of 1% TBA (w/v) in a water bath at 100 °C for 15 min. The absorbance of the resulting solution was measured spectrophotometrically at 532 nm. For the control, buffer was added instead of antioxidants. In the blank tube, the DR was substituted by buffer. All values were presented as mean ± SD for at least three replicates.

Pyrogallol Assay for Superoxide Radical Anion ($O_2^{\bullet -}$). To examine the $O_2^{\bullet -}$ scavenging activity of GLP, a pyrogallol autoxidation assay was performed (*25*). The reaction mixture contained 1.8 mL of Tris-HCl (0.1 mol/L), 1.0 mL of double-distilled water, and 1.0 mL of GLP solution with various concentrations. After incubation in a 25 °C water bath for 10 min, the mixture was combined with 0.2 mL of pyrogallol. The rate of pyrogallol autoxidation was measured at 320 nm and was recorded as change in absorbance per minute (ΔA/min).

Redox Potential Assay. Redox potentials of different fractions of *G. lucidum* extracts were measured as described by our laboratory previously (*24*). A 2.5 mL aliquot of an appropriate concentration of tested sample was mixed with 2.5 mL of PBS (0.2 M, pH 6.6) and 2.5 mL of 1% $K_3Fe(CN)_6$ solution. After 20 min in a 50 °C water bath, 2.5 mL of 10% potassium oxalate ($K_2C_2O_4$) was added into the mixture, and the solution was centrifuged at 500*g* for 10 min. A volume of 2.5 mL of supernatant was taken out and mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% $FeCl_3$. The absorbance of the mixture solution was measured at 700 nm by a Shimadzu UV-265 FW spectrophotometer.

Lipid Peroxidant Assay. Lipid peroxidant value (POV) was measured according to AOAC methods (*26*). Soybean oil and lard without additives were used as the substrates to evaluate the antioxidant activity of GLP. GLP was dissolved in 1 mL of 95% ethanol and added to the fats (30 g/treatment) at different concentrations. The control sample contained 1 mL of 95% ethanol only. The lipid system was thoroughly homogenized and stored at 60 °C in a water bath for 13 days with occasional stirring by a glass rod.

Lipoxygenase (LOX) Assay. The method of measuring LOX activity was modified from the methods of Block et al. and Li et al. (*24*, *27*). Linoleic acid (1.32 μmol/L) was used as the substrate in a 0.2 mol/L, pH 9.0, phosphate buffer with 0.05% Tween-20 at 25 °C. LOX solution (0.2 mL) with 0.05 mL of sample solutions of various concentrations was incubated in a 25 °C water bath for 4 min. Then 0.8 mL of linoleic acid solution was added, and the mixture was kept in a 25 °C water bath for another 4 min. The reaction was terminated by adding 2.0 mL of ethanol. The absorbance at 234 nm was recorded as a function of time on the Shimadzu UV-265 FW spectrophotometer. For the blank, 2.0 mL of ethanol was added first, and buffer (0.05 mL, 0.01 mol/L, pH 7.0) was added instead of sample solution as the control.

Lipid Peroxidation in Mice Mitochondria and Liver Tissue. Livers from rats (male, Qunming, 18−20 g) were homogenized in 0.15 mol/L KCl (10% w/v) and centrifuged at 400*g* and 4 °C for 15 min. The supernatant was used for in vitro lipid peroxidation assays as described previously (*28*). Briefly, 0.2 mL of liver homogenate was mixed with various concentrations of GLP dissolved in deionized water. The mixture was then incubated in a 37 °C water bath for 1 h. The reaction was terminated by cooling in an ice bath. The MDA level in liver homogenates was used to evaluate oxidation. It was analyzed by a MDA detection kit according to the manufacturer's protocol. In another two groups of experiment, 0.1 mL of 10 mM H_2O_2 or 1 mM $FeSO_4$ was added into the incubation mixture, respectively, to evaluate the iron-chelating ability and H_2O_2-quenching ability of GLP.

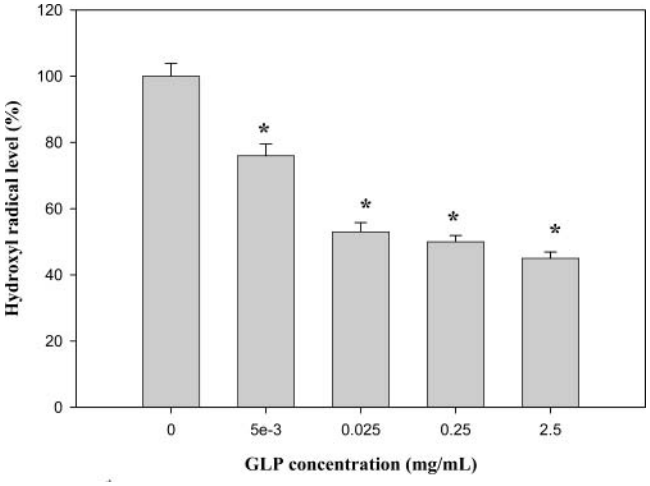

Figure 2. Hydroxyl radical inhibitory activity of GLP in Fe^{3+}–DR system. An asterisk (∗) indicates $p < 0.05$ compared to control group using one-way ANOVA

Mice mitochondria were precipitated by centrifuging 10 mL of 10% liver homogenates at 700*g* and 0 °C for 10 min, followed by centrifuging the supernatant at 7000*g* and 0 °C for 15 min. The resuspended mitochondria fraction (1.0 mL, 0.5 mg of protein/mL) was then mixed with 0.4 mL of $FeSO_4$ (0.5 m mol/L), 0.4 mL of vitamin C (0.5 mmol/L), and 0.4 mL of GLP solution with various concentrations. The mixture was incubated in a 37 °C water bath for 1 h. The reaction was terminated by fast cooling in an ice bath. The MDA level in solution was measured by a MDA detection kit according to the manufacturer's protocol.

Auto-hemolysis and Oxidation of Red Blood Cells. Male rats (Wistar, 190−210 g) were purchased from Hubei laboratory animal center. The rats were housed in stainless steel wire-bottomed cages and acclimated under laboratory conditions (25−28 °C, relative humidity 65%, 12 h light/dark cycle) for 1 week before study. Red blood cells (RBCs) were separated according to the method described previously (*24*). The RBC suspensions were incubated with different concentrations of GLP solution at 37 °C for 24 h. The mixture was then diluted by 0.75% NaCl solution. After centrifugation at 1000*g* for 10 min, the absorbance of the supernatant was measured spectrophotometrically at 540 nm to estimate the auto-hemolysis rate of RBC. The MDA content of the mixture was measured by the MDA detection kit according to the manufacturer's protocol. All values are presented as mean ± SD for at least three replicates.

Statistical Analysis. Results are reported as mean ± SD for at least three analyses for each type of extraction and parameter. Results were subjected to ANOVA and differences between means located using Tukey's multiple-comparison tests run on Minitab release 12 software (State College, PA).

RESULTS

Scavenging Effect of the Hydroxyl Radical. The scavenging effect of GLP on hydroxyl radicals produced in the Fe^{3+}−DR system is shown in **Figure 2**. The hydroxyl radical is an extremely reactive free radical. It causes great damage to living cells due to its ability to react with various molecules such as phospholipids, DNA, and organic acids (*29*). The GLP showed very potent dose-dependent hydroxyl free radical scavenging activity. At a dose of only 0.005 mg/mL, GLP scavenged >20% of hydroxyl free radicals produced. The IC_{50} value (concentration at which 50% of activity is inhibited) of GLP to hydroxyl radical was 0.025 mg/mL.

Different fractions of *G. lucidum* were tested for their scavenging effects on hydroxyl radicals (**Table 1**). Our data suggest that the polysaccharide fraction is not the active component for the free radical scavenging activity of *G. lucidum*.

Table 1. IC_{50} of Water-Soluble Components in *G. lucidum* to •OH

	GLH[a]	crude polysaccharide	crude protein	GLW[b]	GLL[c]	GLP
IC_{50} (mg/mL)	35	950	1.25	0.0 6	0.05	0.025

[a] GLH, higher molecular mass fraction in *G. lucidum*, M > 10000 Da. [b] GLW, water-soluble part in *G. lucidum*. [c] GLL, lower molecular mass fraction in *G. lucidum*, M < 10000 Da.

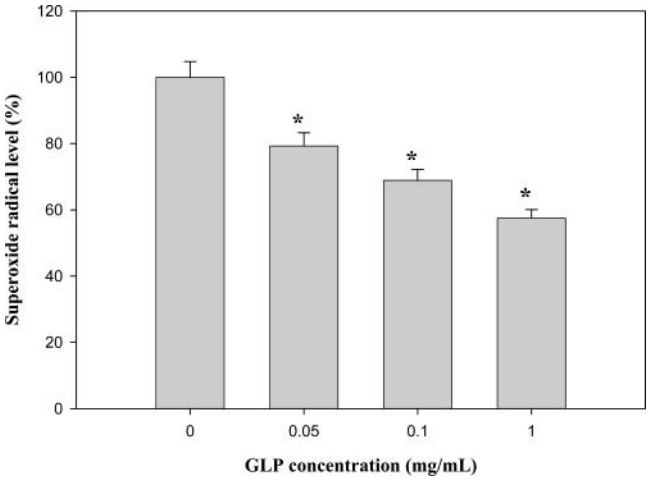

Figure 3. Anti-$O_2^{\bullet-}$ ability of GLP in pyrogallol autoxidation system. An asterisk (∗) indicates $p < 0.05$ compared to control group using one-way ANOVA

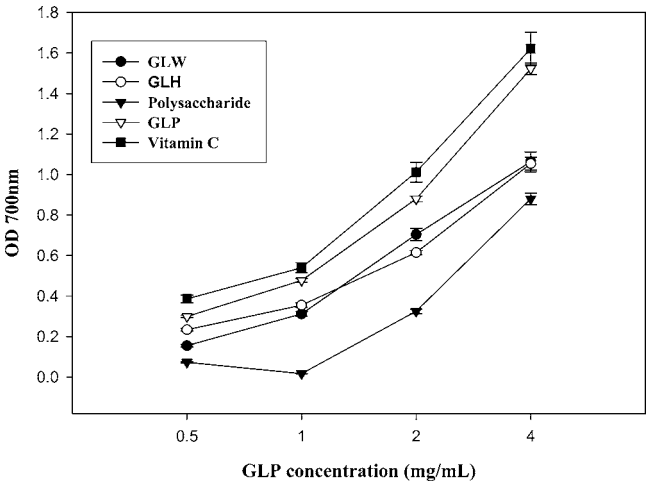

Figure 4. Redox potentials of different components in *G. lucidum*.

The lower molecular mass fraction (M < 10000 Da) is the major bioactive fraction. The protein fraction also possessed weak free radical scavenging activity. The GLP fraction showed the best scavenging effects on hydroxyl radicals with the lowest IC_{50} among the fractions tested.

Scavenging Capacity for Superoxide Radical Anion ($O_2^{\bullet-}$). The pattern of the scavenging effect of GLP on $O_2^{\bullet-}$ is shown in **Figure 3**. At a dose of 0.05 mg/mL, ∼25% of $O_2^{\bullet-}$ produced in a pyrogallol autoxidation system and ∼45% of $O_2^{\bullet-}$ were scavenged by the addition of 1 mg/mL of GLP.

Redox Potential of GLP. The redox potentials of different fractions of the *G. lucidum* water extract were measured. A higher optical density (OD) value means the sample tested possesses a higher reducing power. As shown in **Figure 4**, GLP had the best reducing power among all of the fractions tested,

Table 2. Protection Effect of GLP on the Oxidation of Soybean Oil in Lightproof System at 60 °C[a]

sample	dose (% w/w)	POV (mmol/L kg) 0 days	5 days	8 days	11 days	13 days
control		5.52 ± 0.22	24.20 ± 1.18	34.96 ± 1.72	77.8 ± 3.56	110.7 ± 5.05
BHT	0.01	5.52 ± 0.22	22.20 ± 1.08	29.92 ± 1.45a	59.80 ± 2.58b	87.28 ± 4.19b
GLP	0.01	5.52 ± 0.22	22.70 ± 1.11	27.26 ± 1.32a	58.54 ± 2.49b	86.10 ± 4.08b
BHT	0.05	5.52 ± 0.22	18.40 ± 0.89a	29.40 ± 1.42a	46.40 ± 2.29b	73.68 ± 3.44b
GLP	0.05	5.52 ± 0.22	17.26 ± 0.85a	28.64 ± 1.41a	44.32 ± 2.19b	68.24 ± 3.19b
BHT	0.10	5.52 ± 0.22	17.20 ± 0.85b	24.80 ± 1.21b	35.60 ± 1.74b	51.80 ± 2.44b
GLP	0.10	5.52 ± 0.22	17.06 ± 0.83b	22.02 ± 1.08b	31.86 ± 1.53b	60.00 ± 2.79b
BHT	0.20	5.52 ± 0.22	16.20 ± 0.78b	16.20 ± 0.79b	25.60 ± 1.24b	49.40 ± 2.41b
GLP	0.20	5.52 ± 0.22	15.30 ± 0.735b	19.64 ± 0.94b	22.80 ± 1.09b	43.38 ± 2.06b

[a] Values are the average of three replicate determinations. "a" following an entry indicates $p < 0.05$ compared to control group, and "b" indicates $p < 0.01$ compared to control group using one-way ANOVA.

Table 3. Protection Effect of GLP on the Oxidation of Lard in Lightproof System at 60 °C[a]

sample	dose (% w/w)	POV (mmol/L kg) 0 days	5 days	8 days	11 days	13 days
control		0.48 ± 0.03	6.60 ± 0.36	14.00 ± 0.66	54.20 ± 2.65	83.20 ± 4.22
BHT	0.01	0.48 ± 0.03	5.40 ± 0.25	11.92 ± 0.56a	14.72 ± 0.70b	26.00 ± 1.10b
GLP	0.01	0.48 ± 0.03	6.40 ± 0.35	13.68 ± 0.68	42.22 ± 2.09b	77.52 ± 3.76b
BHT	0.05	0.48 ± 0.03	4.60 ± 0.20a	10.40 ± 0.49b	12.40 ± 0.55b	20.60 ± 0.93b
GLP	0.05	0.48 ± 0.03	5.32 ± 0.22a	12.26 ± 0.59b	38.64 ± 1.89b	64.68 ± 3.19b
BHT	0.10	0.48 ± 0.03	3.40 ± 0.18b	6.96 ± 0.31b	8.04 ± 0.38b	13.84 ± 0.66b
GLP	0.10	0.48 ± 0.03	4.00 ± 0.18b	10.64 ± 0.54b	34.58 ± 1.70b	60.40 ± 2.94b
BHT	0.20	0.48 ± 0.03	3.28 ± 0.17b	4.88 ± 0.22b	7.08 ± 0.33b	11.8 ± 0.43b
GLP	0.20	0.48 ± 0.03	3.50 ± 0.16b	9.44 ± 0.45b	30.40 ± 1.49b	56.66 ± 2.83b

[a] Values are the average of three replicate determinations. "a" following an entry indicates $p < 0.05$ compared to control group, and "b" indicates $p < 0.01$ compared to control group using one-way ANOVA.

and its redox potential was similar to that of vitamin C. The crude polysaccharide fraction had the lowest redox potential.

Antioxidant Activities of GLP in Lightproof Soybean Oil and in the Lard System. The antioxidant effect of GLP was evaluated in the lightproof soybean oil system and in the lard system. In the soybean oil system, addition of GLP significantly reduced peroxide levels in soybean oil over the 13-day duration of the experiment (**Table 2**). The protective activity of GLP was dose-dependent. At a dose of 0.10% (w/w), ~50% of peroxidation was inhibited. Interestingly, the antioxidant activity of GLP in the soybean oil system was comparable to that of the synthetic antioxidant BHT at the same dosage.

In the lard system, GLP showed a dose-dependent inhibition of peroxidation (**Table 3**). Approximately 30% of peroxide production was inhibited by GLP at a dose of 0.20% (w/w). However, BHT showed an 80% inhibition at a dose of 0.20% (w/w).

LOX Inhibitory Activity of GLP. The effect of GLP on LOX activity is shown in **Figure 5**. Clearly, GLP significantly inhibited LOX-catalyzed oxidation of linoleic acid in a dose-dependent manner ($p < 0.05$) with an IC_{50} value of 27.1 μg/mL. Even at the low dose of 0.0085 mg/mL, GLP inhibited 40% of the LOX activity, whereas at a dose of 0.3 mg/mL, GLP inhibited ~90% of LOX activity in vitro.

Effects of GLP on the MDA Content in the Mouse Liver Tissues. Mouse liver tissue was used to evaluate antioxidant activities of GLP. As shown in **Table 4**, GLP inhibited MDA formation in liver tissue homogenates in a dose-dependent manner. MDA is a widely used marker of the oxidation. At the doses tested (ranging from 0.035 to 0.7 mg/mL), GLP inhibited MDA formation in liver tissue by 52−63%. Fe^{2+} and H_2O_2 are

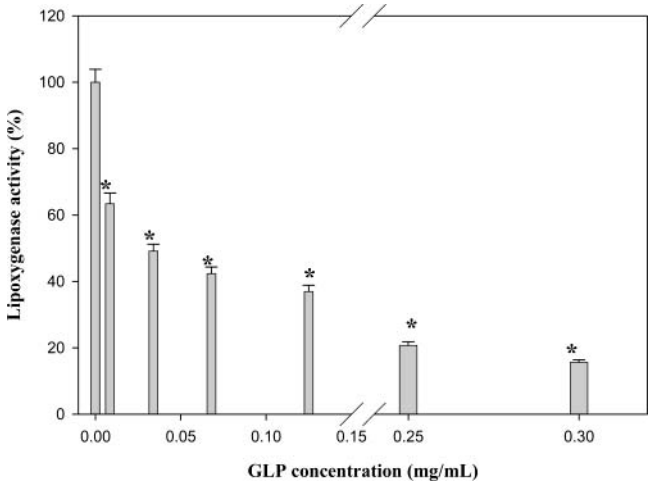

Figure 5. Effect of GLP on lipoxygenase activity in vitro. An asterisk (*) indicates $p < 0.05$ compared to control group using one-way ANOVA

two inducers of free radicals. When the mice liver tissue was incubated with Fe^{2+} or H_2O_2, the MDA content increased significantly ($p < 0.05$). It was found that in the presence Fe^{2+} or H_2O_2, GLP could still inhibit liver tissue MDA levels in a dose-dependent manner. The percentages of MDA formation inhibited by GLP were very similar under different experimental conditions. However, the content of MDA in the system was much higher when Fe^{2+} or H_2O_2 was involved.

Effects of GLP on the Swelling of Mouse Liver Mitochondria and the MDA Content in Mouse Liver Mitochondria. Under experimental conditions, the intact membrane structure of the mitochondria was destroyed by the oxidation of phos-

Table 4. Effect of GLP on MDA Formation of Mice Liver under Incubation with Fe^{2+} and H_2O_2 in Vitro ($n = 10$, $X \pm SD$)[a]

		incubation		incubation with Fe^{2+}		incubation with H_2O_2	
sample	dose (mg/mL)	MDA (nmol/mg)	inhibition ratio (%)	MDA (nmol/mg)	inhibition ratio (%)	MDA (nmol/mg)	inhibition ratio (%)
control	0	356 ± 24.92		681.0 ± 74.91		637.0 ± 57.33	0
GLP	0.035	167.4 ± 15.49a	52.98	300.1 ± 29.77a	55.93	289.2 ± 29.01a	54.60
	0.070	159.8 ± 16.31a	55.11	288.6 ± 27.19b	57.62	263.7 ± 27.44b	58.60
	0.350	144.2 ± 13.27b	59.49	264.2 ± 27.12b	61.20	249.3 ± 27.31b	60.86
	0.700	132.7 ± 11.36b	62.72	244.5 ± 23.64b	64.10	175.9 ± 15.09b	72.39

[a] Values are the average of 10 replicate determinations. "a" following an entry indicates $p < 0.05$ compared to control group, and "b" indicates $p < 0.01$ compared to control group using one-way ANOVA.

Table 5. Effect of GLP on the MDA Content in the Mouse Liver Mitochondria[a]

group	dose (mg/mL)	content of MDA (nmol/mg)	MDA inhibition (%)	swelling inhibition (%)
control	0	7.89 ± 0.02	0	0
GLP	0.01	6.83 ± 0.02	13.4	27.3
	0.025	6.71 ± 0.03b	14.9	43.9
	0.05	6.21 ± 0.01a	21.3	50.7
	0.10	5.59 ± 0.03a	29.1	79.1
	0.25	5.22 ± 0.02a	33.9	85.2
	0.55	5.09 ± 0.02a	35.4	88.6
	1.00	2.73 ± 0.01a	65.4	90.5

[a] Values are the average of three replicate determinations. "a" following an entry indicates $p < 0.05$ compared to control group, and "b" indicates $p < 0.01$ compared to control group using one-way ANOVA.

Table 6. Effect of GLP on Rat Red Blood Cell Auto-hemolysis[a]

sample	dose (mg/mL)	inhibition of the hemolysis of rat RBC (%)	inhibition of the MDA formation of rat RBC (%)
control	0	0	0
GLP	0.010	9.07 ± 0.32a	55.39 ± 1.94b
	0.025	33.22 ± 1.30b	55.70 ± 2.17b
	0.050	49.05 ± 1.52b	60.91 ± 1.89b
	0.100	55.29 ± 2.38b	61.85 ± 2.66b
	0.250	62.33 ± 2.74b	91.24 ± 4.02b
	0.500	60.34 ± 3.32b	83.73 ± 4.61b

[a] Values are the average of three replicate determinations. "a" following an entry indicates $p < 0.05$ compared to control group, and "b" indicates $p < 0.01$ compared to control group using one-way ANOVA.

pholipid bilayers, which resulted in swelling. Moreover, the oxidation of phospholipid membranes resulted in MDA level increases. With the addition of GLP into the system, both the MDA level and the swelling level of the mitochondria were reduced significantly in a dose-dependent manner ($p < 0.05$; **Table 5**). GLP reduced MDA levels by 65.4% at the highest dose tested. However, only 0.05 mg/mL GLP was needed to reach 50% inhibition of mitochondria swelling. Nearly all of the swelling was prevented by adding 1.0 mg/mL of GLP.

Effects of GLP on the Hemolysis of Rat RBC and the MDA Content in Rat RBC. Research shows that RBCs undergo auto-hemolysis daily in vivo. The in vitro model used here was designed to mimic this situation. RBCs undergo hemolysis due to membrane destruction caused during their oxidation. Results show that GLP prevented RBCs hemolysis and MDA formation in a dose-dependent manner (**Table 6**). At doses ≥0.025 mg/mL, GLP prevented the oxidation of RBC significantly ($p < 0.01$).

DISCUSSION

G. lucidum has been used for over 2000 years in traditional oriental medicine and has been linked with numerous pharmacological effects such as anticancer, antiviral, immunomodulating potential, and antihypertension (*30*−*33*).

Soybean oil and lard are two frequently consumed oils, and they have been widely used as oxidation substrates for evaluating antioxidants in lipid systems. Mau et al. have found that among four commonly used medicinal mushrooms, *G. lucidum* showed excellent antioxidant activity in a lipid system. At a dose of 0.6 mg/mL, *G. lucidum* inhibited 2.30−6.41% of lipid peroxidation (*5*). Our experimental results showed that GLP is a very potent antioxidant with high antioxidant activity comparable to that of the synthetic antioxidant butylated hydroxytoluene (BHT) in soybean oil. GLP showed better antioxidant activity in soybean oil than in lard. After a 13-day treatment, the inhibitory effect of only 0.2% GLP on the oxidation of soybean oil was almost 60% compared to the control group. The inhibition rate was 30% in the lard system under the same conditions. The less potent antioxidant activity of GLP in the lard system may be due to the high content of polyunsaturated fatty acids in soybean oil. The polyunsaturated fatty acids could serve as substrates for LOX, which has been shown to be inhibited by GLP.

LOX is mainly responsible for catalyzing the oxidation of polyunsaturated fatty acids with the *cis*,*cis*-1,4-nonconjugated double-bond structure. Research shows that ROS could be produced through the LOX pathways of arachidonic acid metabolism. These reactive forms of oxygen and other arachidonic acid metabolites may play an important role in inflammation and tumor promotion (*34*). A key step for LOX activation is the binding of a non-heme-iron at its active site. The inhibitory activity of GLP might be due to its iron-chelating activity.

The activities of GLP in preventing lipid oxidation in soybean oil and lard systems might be due to its ability to react with peroxy radicals and, thus, terminate lipid peroxidation chain reactions. ROS are important causative agents for a number of human diseases, including cancer, arteriosclerosis, and aging-related diseases. It has been shown that $^{\bullet}OH$ is the major active oxygen species causing lipid oxidation (*35*). GLP showed potent activities to scavenge two of the most reactive oxygen species, $^{\bullet}OH$ and $O_2^{\bullet-}$, in a dose-dependent manner. Therefore, the antioxidant activity of GLP may also be partly due to its ability to scavenge both $^{\bullet}OH$ and $O_2^{\bullet-}$.

GLP showed a higher antioxidant activity in an induced oxidation system than in a noninduced oxidation system using mouse liver homogenates (**Table 4**). The inhibition rate of 0.7 mg/mL GLP on the H_2O_2-induced MDA formation was 72%, significantly higher than in the same system without H_2O_2 ($p < 0.05$). Also, GLP was more potent in the Fe^{2+}-induced oxidation system than in the same system without inducers. These data showed that GLP is a potent scavenger of peroxyl

radicals, as well as an excellent iron chelator. Phospholipids are major constituents of biological membranes. They could be easily attacked by ROS produced in biological systems, and the oxidation could result in serious disorders. Aqueous extracts of *G. lucidum* (1.5% w/v) have been shown to have protective effects on the cell membrane oxidation in situ (*11*). As demonstrated in vitro (**Table 5**), GLP has a strong protective activity against mouse mitochondrial swelling. The swelling was mainly due to the oxidation and dysfunction of the phospholipid bilayer of the mitochondria. Because GLP showed potent free radical scavenging activity, it is reasonable to hypothesize that GLP exerted its protection against phospholipid oxidation through its strong radical scavenging activity.

In summary, we propose that the antioxidant activity of GLP may be due to the following mechanisms: (1) free radical scavenging activity, whereby excessive free radicals that are generated during the course of normal biological metabolism are quenched via GLP, thus preventing oxidation of unsaturated fatty acids and the destruction and dysfunction of the cellular membrane; (2) low redox potential (GLP could react with free oxygen or ROOH to block the chain reaction of lipid oxidation); and (3) metal chelating activity.

The antioxidant activities of *G. lucidum* have been documented by many researchers (*1*, *5*, *6*). In a human intervention study, ingestion of a *G. lucidum* supplement was found to cause an acute increase in plasma antioxidant capacity but no significant changes in any biomarkers of antioxidant status in healthy, well-nourished human subjects (*6*, *36*). Hot-water extracts of *G. lucidum* have shown radioprotective activity and protection against DNA damage in vitro. The water-soluble polysaccharides were associated with these protective properties (*37*). A *Ganoderma* polysaccharide peptide (GLPP) showed potent protection effects against copper-induced low lipoprotein oxidation in vitro, as well as alloxan-induced oxidative stress in vivo (*38*). An amino-polysaccharide fraction (G009) from *G. lucidum* showed similar antioxidant activities against iron-induced lipid oxidation in vitro, as well as inactivation of hydroxyl radicals and superoxide anions (*8*). Mau et al. showed that the phenolics in the methanol extract of *G. lucidum* are the antioxidant components (*5*). Our results, for the first time, showed that the low molecular weight water extracts from *G. lucidum* have better hydroxyl radical scavenging activity than the high molecular weight fractions. Importantly, we have shown that the peptides (GLP) were the major antioxidant components. On the basis of all of this information, it seems that the antioxidant components are different depending on the extraction method. We may be able to utilize *G. lucidum* more efficiently by optimizing the extraction procedures to retain all of its bioactive components.

ROS are constantly generated for various physiological functions in the human body. The excessive production of ROS is generally overcome by the body's own antioxidant systems (*39*, *40*). An imbalance caused by excessive oxidants may result in oxidative damage to many large biomolecules, such as lipids, DNA, and proteins (*39*). Our data showed that the GLP could effectively scavenge superoxide radicals, hydroxyl radicals, and lipid peroxides, which are the major forms of ROS generated in the human body. Thus, GLP might serve as a promising agent to reduce oxidative damage to biomolecules by modulating the effects of reactive oxidants.

Oxidative stress is associated with an increased risk of degenerative diseases, such as cardiovascular diseases and cancer (*41*, *42*). It has been well documented that *G. lucidum* has various health benefits in animals and humans, such as hepatoprotective effects, anticancer activity, and cardiovascular protective effects (*43*−*45*). Polysaccharides, triterpenoids, polysaccharide-protein, and polysaccharide-peptide have been shown to be the anticancer components of *G. lucidum* (*19*, *46*−*48*). Many researchers have shown that water-soluble polysaccharides or their derivatives could inhibit tumor growth by modulating the immune system (*7*, *49*, *50*). Lee et al. proposed that the hypotensive action of *G. lucidum* was due to its central inhibition of sympathetic nerve activity (*45*). Our data suggest *G. lucidum* might exert its anticancer and hypotensive activities through another approach by quenching excessive free radicals and attenuating the oxidative stress. ROS is involved in the cascades of carcinogenesis (*51*−*53*). Also, there is compelling evidence that oxidative stress plays an important role in triggering the complex cascades leading to atherosclerosis (*54*, *55*). ROS have been well documented as critical signaling molecules in various cellular events involved in cardiovascular disease development, especially in areas focused on angiotensin II (AngII) (*56*). Thus, ROS scavenging compounds might serve as promising agents in the prevention of cardiovascular diseases and cancer. GLP showed potent ROS scavenging activities in various chemical and biological systems tested. Therefore, it is reasonable to conclude that GLP could act as a potential chemopreventive agent against cancer and cardiovascular diseases. Further studies on the antioxidant activities of GLP in vivo are necessary to assess the pharmacological values of GLP as chemopreventive agents.

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