In Vitro Antioxidant and Anti-Inflammatory Activities of Protocatechualdehyde Isolated from *Phellinus gilvus*

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(Received June 22, 2010)

Summary Although various biological activities of *Phellinus gilvus* (PG) have been reported, the active compounds responsible for these effects are not known. Here, we evaluated the activity of various solvent extracts of PG, and found the ethyl acetate extract (Fd) to be the most active fraction, showing a strong DPPH free radical scavenging activity, and inhibitory effects on LPS-induced nitric oxide (NO) production and COX-2 mRNA expression in RAW264.7 macrophages. Six major compounds were identified from the ethyl acetate extract of PG, and protocatechualdehyde (PCA) was supposed to be the major phenolic compound of PG responsible for its DPPH free radical scavenging activity and its inhibitory effects on LPS-induced NO production in RAW264.7 cells. Further in vitro and in vivo experiments are currently underway to confirm this observation and to investigate the detailed molecular mechanisms involved in the process as well as the biological activities of other fractions of Fd.

Key Words *Phellinus gilvus*, protocatechualdehyde, DPPH, NO, RAW264.7 cells

*Phellinus gilvus* (PG) is a medicinal mushroom belonging to *Hymenochaetaceae* basidiomycetes, and has advantages over many *Phellinus* species due to its short growth period (3 mo), making it cheaper to produce (1–3). Previous studies in our laboratory and by other investigators have established various biological activities of PG. Among the reported benefits of PG are inhibition of pulmonary inflammation, prevention of intraperitoneal adhesion under infectious circumstances, promotion of dermal wound healing, anti-platelet aggregation and antitumor activities (1–4). In a previous study with various solvent extracts of PG, we have demonstrated marked DPPH free radical scavenging and xanthine oxidase (XO)-inhibitory activities of the ethyl acetate extract of PG, along with the enhancement of cellular immunity by hot water extracts of PG and inhibition of thrombin-induced platelet aggregation by chloroform-, methanol- and butanol extracts of PG (3). In the current investigation, we determined the major components of the ethyl acetate extract of PG responsible for its biological activities and further compared the magnitude of the antioxidant/anti-inflammatory activities of components with the various fractional extracts of PG.

The fruiting body of PG was provided by Gyeongbuk Agriculture Technology Administration (Daegu, Korea). The preparation of PG fractional extracts was reported earlier (3). Briefly, fruiting bodies of the mushroom were cut into small pieces and dried at 40–50°C for 48 h. The pieces were homogenized, extracted with water (1 : 25) at 100°C for 10 h, and the aqueous phase (Fa) concentrated at 80°C in a rotary evaporator (Büchi Rotavapor R-114, Switzerland) and the final volume reduced to 1/10 of the initial volume. The Fa was then mixed with 95% ethanol (1 : 3, v/v) and stored at 4°C overnight. The solution was then centrifuged at 15,000 × g for 30 min, the precipitate dialyzed (1 : 100,000) in water, filtered, centrifuged, re-dissolved and lyophilized to obtain the polysaccharides (Fc). The supernatant was evaporated to remove the ethanol and extracted with ethyl acetate (4 vol.). The upper ethyl acetate layer (Fd) and the lower aqueous phases (Fc) were then evaporated and lyophilized.

Gas chromatography–mass spectrometry (GC-MS) analysis was performed to detect components of Fd. The instruments were composed of HP 6890 GC (Hewlett Packard, Waldbronn, Germany) and HP 5974 MS apparatus with an HP-5 MS column. One microliter of sample was injected in a split mode (50 : 1). Helium gas was used as a carrier at a rate at 0.7 mL/min. Initial GC oven temperature was 70°C, which was then adjusted to 300°C for 10 min at a rate of 7°C/min. The injector and detector temperatures were set to 200°C and 300°C, respectively. The compounds were identified by comparison of their mass spectrometric fragmentation.
patterns with specific standards. Six major compounds of Fd fraction were detected using GC-MS analysis. These include: protocatechualdehyde (PCA) (55.64%), 1,3-diazabicyclo [3.1.0] hexane (12.45%), maltol (9.73%), propane-2-13C (8.56%), tridecanoic acid (8.17%), and azelaic acid (5.45%) of the total detectable compounds. Due to its highest proportion in Fd, and about 8.7% of the dry weight of the Fd fraction.

We characterized the biological activities of the PCA isolated from Fd, along with other fractional extracts of PG. Mouse RAW264.7 macrophages (Korean Cell Line Bank, Seoul, Korea) were used in this study. Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin. Cultures were maintained at 37°C in a 5% CO₂ humidified atmosphere. First, we determined the antioxidant-activity of PCA and other fractional extracts using the DPPH free radical scavenging assay by following a previously described method with slight modifications (8). Briefly, 10 µL of test samples or standard was added to 200 µL of DPPH in ethanol solution (100 µM) in a 96-well microplate (SPL, Suwon, Korea). After incubation at 37°C for 30 min, the optical density (OD) of each well was measured at 517 nm using VERSA max microplate reader (Molecular Devices Corp., Sunnyvale, CA). Scavenging activity of samples on DPPH free radical was calculated according to the following formula: Inhibition (%)=(1−((Sd−Se)/C))×100%, where Sd is the OD of sample with DPPH, Se is the OD of sample with ethanol, and C is the OD of the vehicle with DPPH. IC₅₀ value is the concentration of the sample required to scavenge 50% of DPPH free radicals. As shown in Fig. 1, PCA showed a dose-dependent scavenging of DPPH free radicals with an IC₅₀ value of 10.86 µg/mL, which was significantly greater than that of ascorbic acid and other fractional extracts of PG. Our quantitative studies revealed that the amount of PCA in the Fd fraction was less than 10%.

However, its DPPH scavenging activity was almost five-fold greater than that of the Fd fraction. This suggests that PCA may be the major active compound in the Fd fraction that accounts for the majority of the scavenging activity of Fd. PCA had also been isolated from other mushrooms, such as Phellinus linteus (9), and consistent with our findings, the antioxidant activity of this compound has been documented. For example, PCA isolated from the fruits of greater cardamom (Annamom subulatum) had a stronger DPPH scavenging activity than such natural antioxidants as alpha-tocopherol and l-ascorbic acid (6). Previous studies have established phenolic compounds as major antioxidants of medicinal plants, mushrooms, essential oils, spices, fruits, and vegetables (10, 11). In a recent report, Kim et al. identified a total of 28 phenolic compounds from five edible and five medicinal mushrooms commonly cultivated in Korea (11). The average total DPPH radical scavenging activities of methanol extracts (10 mg/mL) of the 10 mushrooms species ranged between 10 and 72%. The DPPH scavenging activity of both Fd and Fc of PG (10 mg/mL, >95%) was higher than in the previous study. The differences may be attributed partly to differences in the mushroom species or the content and concentration of phenolic compounds.

Increased nitric oxide (NO) production is a key feature of inflammation, specifically in bacterial infection and LPS-induced pathologies (12, 13), and therapeutic agents that inhibit excessive NO production may be useful in the prevention and treatment of these conditions. In a previous study, we demonstrated the potential of a hot water extract of PG to reduce LPS-induced NO production in RAW264.7 macrophages (3). Herein, we further investigated the NO-inhibitory activities of PCA extracted from Fd, along with other fractional extracts of PG. RAW264.7 cells (2×10⁵ cells/mL) were pretreated with PCA or various fractions of PG at different concentrations for 30 min, and then treated with LPS...
PCR analysis, RA W264.7 cells (4 and 5 COX-2: 5 sense).

There were no differences in NO levels in RAW264.7 macrophages treated with PCA or various fractions of PG compared with untreated cells, while LPS (0.5 μg/mL) induced significantly higher levels of NO in RAW264.7 cells (data not shown). As shown in Fig. 2, both PCA and various fractions of PG showed dose-dependent inhibition of LPS-induced NO production in RAW264.7 cells. Among the fractional extracts of PG, Fd had the greatest inhibitory activity with an IC₅₀ value of 36.70 μg/mL, whereas Fb showed the lowest activity. PCA had even greater activity of NO inhibition than Fd with an IC₅₀ value of 19.46 μg/mL, indicating its possible contribution for the observed activity of the Fd fraction of PG.

NO production is usually associated with iNOS gene expression. Therefore, we performed reverse transcription PCR (RT PCR) analysis to evaluate the effect of PCA and Fd on LPS-induced iNOS mRNA expression in RAW264.7 cells. COX-2 is an important enzyme induced by inflammatory stimuli such as cytokines, and is responsible for the production of prostaglandins that contribute to the pain and swelling during inflammation (14). To gain additional insight into the anti-inflammatory effects of PCA and Fd, we also evaluated the expression of COX-2 together with iNOS. For the RT PCR analysis, RAW264.7 cells (4×10⁵ cells/mL) were pretreated with PCA or Fd at various concentrations (0–100 μg/mL) for 30 min, and then treated with LPS (0.5 μg/mL) for an 8-h incubation period, total RNA was isolated using the Trizol reagent according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). One microgram of RNA was incubated with 1.5 μL oligo-dT₁₅ (20 pmol) and 20 μL DEPC-DDW at 70°C for 10 min and then cooled on ice. Each sample was reverse-transcribed to cDNA using an AccuPower RT PreMix (Bioneer, Daejeon, Korea). The cDNA products were subjected to 30 cycles of PCR amplification in the presence of specific sense and antisense primers using AccuPower PCR PreMix (Bioneer). Each cycle consisted of denaturation at 94°C for 45 s, annealing at 50 to 60°C for 45 s, and extension at 72°C for 45 s. Listed below are the primer sequences used in this study: iNOS: 5'-CCCTTCGAAGTTTCTGGCAGCAG-3' (sense) and 5'-GCTGTCAGGCTCCTGCTGTTTG-3' (antisense). COX-2: 5'-CCTACATCTGACAGCAGCTTCTT-3' (sense) and 5'-ATGCTTTGAGTTATGTTG-3' (antisense). β-actin: 5'-ATGCTTTGAGTTATGTTG-3' (sense) and 5'-GGAGGAAAGAGCTGCCAGAT-3' (antisense).

Figure 3 shows the effect of PCA or Fd fraction of PG on iNOS and COX-2 mRNA expression. As shown in the figure, the mRNA expression of iNOS or COX-2 was nearly undetectable in the absence of LPS. However, LPS-stimulation markedly increased the expression of both iNOS and COX-2 genes. As shown in the upper panel of Fig. 3, Fd inhibited the effect of LPS in a concentration-dependent manner. Similarly, a concentration-dependent inhibition of LPS-induced iNOS and COX-2 mRNA expression was observed for PCA, indicating its possible contribution for the observed anti-inflammatory activity of Fd. The transcriptional factor nuclear factor κB (NF-κB) and the mitogen-activated protein kinase (MAPK) are important regulators of inflammatory mediators, including NO, and pro-inflammatory cytokine expression (15, 16). Several antioxidant phytochemicals modulate redox-sensitive pathways, including inflammatory and apoptosis pathways via downstream signaling components, such as kinases, phosphatases, and transcription factors (17, 18). For example, antioxidant phytochemicals suppressed LPS-induced iNOS gene expression in macrophages by inhibiting NF-κB activation (16). Phenolic compounds with antioxidant property have also shown strong COX-2-inhibitory and anti-inflammatory activities (19). Therefore, it is likely that the observed suppression of iNOS and COX-2 by PCA may be associated with its antioxidant activity as revealed in its scavenging effect on DPPH free radicals. However, this should be validated in further studies that address the effect of PCA or Fd on the detailed molecular mechanisms involved in LPS-induced inflammatory response in macrophages.

We performed an in vitro cell viability assay to rule out the possibility that the NO-inhibitory activity of PCA or fractional extracts of PG might be associated with cell deaths caused by PCA or PG extracts. For the MTT assay, RAW264.7 cells (5×10⁴ cells/mL) were treated with PCA or PG extracts for 24 h. Thereafter,
the media were replaced with new media before adding 10 μL MTT solution (2 mg/mL) into each well. After a 4-h incubation period, supernatants were aspirated and the insoluble formazan product dissolved in 200 μL DMSO. The OD of the culture wells was measured using VERSA max microplate reader at 570 nm. As depicted in Fig. 4, no cytotoxicity was associated with any fractional extract of PG at the range of concentrations tested. Similarly, PCA had no cytotoxic effect except for the highest concentration tested (100 μg/mL), where 20–22% loss of cell viability was observed compared to untreated controls. This effect may be considered insignificant because the concentration of PCA required to inhibit LPS-induced NO production was much more lower (an IC50 value of 19.46 μg/mL) than its cytotoxic level.

Finally, the present study focused on the antioxidant and anti-inflammatory activities of PCA, the main component of Fd of PG. However, we also identified small proportions of five other compounds. The biological activities of some of these compounds were previously reported. For example, tridecanoic acid, a saturated fatty acid, has shown antioxidant activity at a concentration of 60 ppm using liposome oxidation assay (20). However, the same authors found that no COX-inhibitory activity (both COX 1 and 2) was observed for tridecanoic acid tested at a higher (100 ppm) concentration. Maltol, another component of Fd, has also shown a low NO-scavenging activity with an IC10 value of 33.7 μg/mL (21). Bactericidal activity of azelaic acid, a therapeutically active agent widely used in dermatology, has been established, although the mechanisms of the reported activity remains to be confirmed (22). To the best of our knowledge, reports on the biological activities of 1,3-diazabicyclo [3.1.0] hexane and propane-2-13C, other components of Fd, are scarce or not available. Although our results suggest that PCA may be the major phenolic compound of Fd of PG responsible for the observed antioxidant and anti-inflammatory activities, this should be confirmed by further studies. To this end, both in vitro and in vivo experiments are currently underway to investigate the detailed molecular mechanisms for the observed activities of PCA as well as the biological activities of other fractions of Fd.

Acknowledgments
This study was supported in part by Technology Development Program for Agriculture and Forestry, Ministry for Agriculture, Forestry and Fisheries and Application Center Program, Ministry of Science and Technology and in part by Ministry of Commerce, Industry Energy (MOCIE) through the Center for Traditional Microorganism Resources (TMR) at Keimyung University.

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