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Analytical Methods

Effect of ultrasonic extraction conditions on antioxidative and immunomodulatory activities of a *Ganoderma lucidum* polysaccharide originated from fermented soybean curd residue

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ABSTRACT

A crude *Ganoderma lucidum* polysaccharide (GLPL) was extracted from fermented soybean curd residue by ultrasonic assisted extraction. The optimal extraction conditions were 30 min at 80 °C with 80 W and water to solid ratio of 10, and with this method 115.47 ± 2.95 mg/g of GLPL yield was obtained. Additionally, the antioxidant and immunomodulatory activities of GLPL were investigated. The results showed that GLPL exhibited strong antioxidant effects, which included scavenging activities against DPPH radicals, hydrogen oxide and ABTS radicals with IC_{50} values of 0.23, 0.48 and 0.69 mg/mL, respectively. For immunomodulatory activities, GLPL was shown to strongly stimulate the proliferation of macrophages (158.02 ± 13.12%), the production of nitric oxide and phagocytosis (21.16 ± 1.65 μM), and, at 40.00 μg/mL, protected macrophage from Doxorubicin (DOX) (0.16 ± 0.003).

1. Introduction

Ganoderma lucidum (*G. lucidum*), a medicinal fungus called "Lingzhi" in China is one of the most famous fungi in traditional Chinese medicine. In regions of China and other Asian countries, *G. lucidum* has been used as a remedy to promote health and longevity (Yuen & Gohel, 2008). Modern pharmaceutical research shows that *G. lucidum* polysaccharide (GLPL) has some physiological effects, including strong antioxidant, immunomodulating (Lin et al., 2006), and anti-tumour activities (Yuen & Gohel, 2008).

Traditionally, polysaccharides are extracted from *G. lucidum* fruiting bodies; however, the incubation time of these fruiting bodies is as long as 60 days with a polysaccharide yield of 32.7 mg/g (Huanga & Ninga, 2010). In this study, GLPL was produced using the food waste from soybean curd residue (SCR), which could greatly reduce the production time. Therefore, this is a promising new technology for GLPL production.

SCR, a by-product of tofu, soymilk or soy protein processing, is discharged as an agro-industrial waste and has caused severe environmental pollution. In fact, SCR is rich in fat, starch, protein and sugar and could be used as a high quality media for microbial fermentation (Shi, Yang, Guan, Wang, & Zhang, 2012a,b). However, there are few previous reports describing the production of GLPL using SCR, and GLPL from natural food waste could be used as a functional food additive in the future.

For the present, several conventional extraction techniques have been reported for the extraction of GLPL. Hot water technology is the main and most conventional extraction method for polysaccharides mentioned in recent studies (Guan, Zhang, Yang, Xin, & Liu, 2011; Kazahiro, Akira, Naomi, & Hidenori, 2009; Shi et al., 2012a,b). However, it is usually associated with a longer extraction time, more than 2 h, and a higher temperature (95 °C) but a lower extraction efficiency with a yield of less than 70.00 mg/g (Xiangqun, Yongde, & Hui, 2011). Recently, ultrasonic assisted extraction has been employed widely in the extraction of target compounds from different materials because of the facilitated mass transfer between immiscible phases through super agitation at a low frequency. It offers the benefits of high reproducibility, shorter times, simplified manipulation, and lowered energy input and solvent consumption (Khan, Maryline, Fabiano-Tixier, Dangles, & Chemat, 2010).

Until now, there has been no comprehensive evaluation of the antioxidant and immunomodulatory activities of GLPL. Papers report that polysaccharides from mushrooms can enhance and activate macrophage-based immune responses, leading to immunomodulation, anti-tumour activity, wound-healing and other therapeutic effects (Huanga & Ninga, 2010; Shi et al., 2012a,b). Macrophages have a significant role in host defence mechanisms. Phagocytic activity produces reactive oxygen species (ROS) and

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nitric oxide (NO) in response to stimulation from a variety of agents, and it can inhibit the growth of a wide variety of tumour cells and micro-organisms (Schepetkin & Quinn, 2006). Moreover, the immunomodulatory activity not only involves effects macrophage activation but also proliferation and differentiation of these cells (Schepetkin & Quinn, 2006).

Reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), hydroxyl radical (HO·) and other free radicals, are by-products of biological metabolism (Xu et al., 2009). In recent years, many studies have shown that ROS may be responsible for or contribute to human diseases (Liu, Wang, Pang, Yao, & Gao, 2010; Wu & Hansen, 2008; Xu et al., 2009). Antioxidants can scavenge free radicals and protect against diseases. Bioactive components in fruits and vegetables have been shown to have beneficial activity (Wu & Hansen, 2008; Xu et al., 2009). In recent years, there has been increasing evidence that some polysaccharides isolated from plants, herbs and fungi also have putative health benefits and low cytotoxicity (Chatchai, Saranyu, Khajeelak, Chantragan, & Rakrudee, 2011; Maja, Klaus, Dragica, Johannes, & Leo, 2011).

The purpose of this study was to extract GLPL using an optimal ultrasonic assisted extraction method from fermented SCR using *G. lucidum*, and to verify its antioxidant activities and immunomodulatory activities *in vitro*.

2. Materials and methods

2.1. Materials and chemicals

Eagle's minimum essential medium (MEM), 2,2-diphenyl-1-picry-hydrazyl radical (DPPH), fetal bovine serum (FBS), phosphate buffered saline (PBS), trichloroacetic acid (TCA) and 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) were purchased from Sigma–Aldrich, Inc. (Saint Louis, MO, USA). Lipopolysaccharide (LPS) from *Escherichia coli* 055, ascorbic acid, hydrogen peroxide, chloride ferric, ferrous sulphate, trichloroacetic acid, sodium salicylate and ethylenediaminetetraacetic acid (EDTA) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The SOD Assay Kit-WST and Cell Counting Kit-8 (CCK-8) were purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). Doxorubicin (DOX) was purchased from TopoGEN, Inc. (Florida, USA). All other chemical reagents were of analytical grade.

2.2. Microorganism and culture conditions

The A50 strain of *G. lucidum* was purchased from Agriculture and Forestry Strains in Kaishas, Japan. The strain was maintained on potato dextrose agar (PDA) at 4 °C. 15 mL of the liquid culture was added to a 50-mL flask containing one unit of mycelia agar, which was a 5 mm × 5 mm square that was obtained using a self-designed cutter. The initial pH was from 5.0 to 5.5, and the culture was incubated on a rotary shaker at 100 rpm and 25 °C for 6 days. The seed for the solid culture was obtained from the liquid culture. Solid-state fermentation was performed in a 200-mL flask with wet SCR in optimal culture conditions and incubated at 25 °C. All of the media were autoclaved at 121 °C for 20 min prior to use.

2.3. Cell lines

The murine macrophage cell line, RAW 264.7 was obtained from the Riken Cell Bank (Tsukuba, Japan) and maintained in MEM medium containing 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL of streptomycin at 37 °C in a humidified 5% CO_2 atmosphere (ESPEC CO_2 Incubator). The cells were cultured for 2–3 days to reach logarithmic phase and then used for experiments.

2.4. Preparation of GLPL

The fermented SCR was dried in a convection oven at 50 °C and ground to a powder. The impurities were removed from the crushed powder (1.00 g) with 80% ethanol at room temperature for 24 h. The eluent was discarded and the residue was further extracted using different experimental conditions of ultrasonic assisted extraction. Then, the extract was filtered and centrifuged at 7500 rpm for 30 min at room temperature. The supernatant was concentrated in a rotary evaporator under reduced pressure at 50 °C and free protein layer was removed using the method of Sevage (Staub, 1999). Finally, the extract was subjected to precipitation with fourfold volumes of ethanol. The GLPL curds were collected by centrifugation, washed with ethanol twice, and freeze-dried. Total GLPL was determined using the phenol–sulphuric acid method, and D-glucose was used as the standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The results were expressed as milligram of glucose equivalent per gram of fermented SCR.

2.5. Assay for antioxidant activities of GLPL

HO· scavenging activity, the ferrous metal ion-chelating activity, the reducing power and the ability of GLPL to quench H_2O_2 were measured according to a literature procedure with certain modifications (Liu et al., 2010).

DPPH radical-scavenging activities and the levels of SOD-like activity of GLPL were measured according to the manufacturer's instructions (Shi et al., 2012a,b).

ABTS radical-scavenging activity of GLPL was measured according to the method of Trishna et al. (2011) with slight modifications.

2.6. Immunomodulation activities of GLPL

2.6.1. Bioactivity assay

The effect of GLPL on the proliferation of RAW 264.7 cells was estimated using CCK-8, and the method described by Shi et al. (2012a,b). The data were expressed as percentages of the control.

2.6.2. Measurement of the production of the nitric oxide

The nitrite accumulation was measured using the Griess reagent and used as an indicator of nitric oxide (NO) production in the medium (Shi et al., 2012a,b). $NaNO_2$ was used as a standard to calculate the nitrite concentrations.

2.6.3. Phagocytosis assay

The phagocytic ability of the macrophages was measured using a neutral red uptake assay (Wei, Zhao, Shi-Fei, & Yong-Quan, 2008).

2.6.4. Protective activity

RAW 264.7 cells were cultured in a 96-well plate at a density of 5×10^4 cells/mL for 24 h at 37 °C in a 5% CO_2 atmosphere. Then the cells were incubated with DOX (5.00 μmol/L) in the presence or absence of various concentrations of GLPL for 24 h. After drug exposure, 10.00 μL of CCK-8 solution was added and incubated at 37 °C for 4 h. The cell numbers were quantified by reading the absorbance at 450 nm. The data were expressed as the percentages of the control.

2.7. Statistical analysis

The factors affecting GLPL extraction by ultrasonic assisted extraction are shown in Table 1. According to the orthogonal