Extraction and antioxidant activity of Ganoderma lucidum polysaccharides using electrolyzed oxidizing water

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Abstract. The fruiting body of \textit{Ganoderma lucidum} (Ling Zhi) is a traditional medicinal mushroom for promoting health and life expectancy. \textit{G. lucidum} polysaccharides (GLPs) has several biological activities, including antioxidant, antitumor, antimicrobial and increase the immunity. In order to extract GLPs, electrolyzed oxidizing water (EOW) was used to accelerate their dissolution from the cell walls. Compared to traditional extraction method, electrolyzed oxidizing water is an environment friendly and economically feasible method. The extraction conditions including time, temperature, pH and water-to-solid ratio were optimized by single factor experiments followed by an orthogonal experiment, for maximising total sugar content and antioxidant capacity. The optimal extraction time, temperature, EOW pH and water-to-solid ratio were 4 h, 100°C, pH 2.5 and 20 mL/g, respectively, which yielded 78.47 ± 2.95 mg/g of GLP. The extracted GLP exhibited strong antioxidant effects, including scavenging of DPPH• and HO•, with IC\textsubscript{50} values of 0.55 mg/mL and 0.76 mg/mL, respectively.

1. Introduction
Fungal polysaccharides have recently been proposed as highly promising factors in industrial applications, including bio pharmacy and cosmetology. \textit{Ganoderma lucidum}, an important edible and medicinal fungus, has long been used to promote health and life expectancy, was named as “Ling Zhi” in China. In recent decades, it has become a popular dietary supplement around the world. As a main bioactive ingredient of water-extracts, \textit{G. lucidum} polysaccharides (GLPs) have been isolated and investigated extensively. Modern pharmacological studies shows GLP to have several physiological functions, including strong antioxidant effect, immunomodulating, and anti-tumor activities. As part of the cell wall or in the form of intracellular inclusions, GLPs are difficult to be separated. Therefore, developing a novel and optimized extraction technology is vital in the exploitation of GLP.

Electrolyzed oxidizing water (EOW) has specific physico-chemical and reaction properties. It is a strong oxidizing agent which makes it applicable in many fields of food processing, such as sterilization, enzymatic inactivation and extraction of active substrate. EOW has been evaluated as a more effective extraction method, mainly due to enhanced extraction yield, reduced solvent consumption, and environmental friendliness.

In the current study, based on single factor experiments and followed by an orthogonal experiment, the EOW extraction of GLPs from the fruiting bodies of \textit{G. lucidum} was optimized. In addition, the
antioxidant activities of GLPs were also examined.

2. Materials and methods

2.1. Materials and reagents
The dried fruiting bodies of G. lucidum were collected from Infinitus Co. (Guangdong, China), and crushed into fine powders with a disintegrator (TYS-100, Guangzhou Zhonglan Medical Equipment Co., Ltd., Guangdong, China), sieved (40 mesh) and finally stored in vacuum.

2.2. Electrolyzed oxidizing water (EOW) preparation
An electrolyze kindly supplied by Gelisen Biotechnology Co. (Beijing, China) was used to generate EOW. A 20% NaCl aqueous solution was added to the cathode generator chamber, and a 0.1% NaCl aqueous solution was added to the anode generator chamber. Electric voltage was maintained at 25 V until the desired EOW pH was reached. EOW should be stored in a refrigerator at 4°C.

2.3. Extraction and determination of GLP yield
The extraction was carried out using a series of EOW pH, water-to-solid ratios, temperatures, and extraction times (Table 1). The supernatant was concentrated to approximately 10% of the original volume in a rotary evaporator (RE52CS, Shanghai Yarong Biochemical Instrument Co., Ltd., Shanghai, China).

The supernatant was then treated by the Sevag method for protein removal. The residual portion was mixed with four volumes of dehydrated ethanol (ethanol final concentration, 80%) and stored overnight at 4°C. After centrifugation, the precipitate was washed by anhydrous ethanol for three times, thus the crude GLPs were obtained.

The weight of polysaccharides were obtained by recalculating the carbohydrate content results. The GLP yield of each extraction was calculated by the following equation:

\[
Yield \text{ (mg/g)} = \frac{W_p}{W_m} \times 1000
\]

where \( W_p \) is the weight (g) of GLP, and \( W_m \) is the weight (g) of material.

2.4. Optimization of EOW extraction conditions
The four independent parameters selected were EOW pH, extraction time, extraction temperature, and water-to-solid ratio. The complete factorial design consisted of 9 extractions (Table 2) that were carried out in a random order. A common mathematical procedure was used to calculate and analyze the GLP yield. Triplicate extractions were performed and the average of all three determinations was plotted on a graph.

Table 1. Parameters and levels of the orthogonal experiment.

| Variable                     | Level 1 | Level 2 | Level 3 | Level 4 | Level 5 |
|------------------------------|---------|---------|---------|---------|---------|
| A. temperature (°C)          | 60      | 70      | 80      | 90      | 100     |
| B. extraction time (h)       | 2.0     | 3.0     | 4.0     | 5.0     | 6.0     |
| C. EOW pH                    | 2.5     | 3.5     | 4.5     | 5.5     | 6.5     |
| D. water-to-solid ratio (mL/g)| 10      | 20      | 30      | 40      | 50      |

2.5. Antioxidant activity assays

2.5.1. DPPH• scavenging assay. DPPH• scavenging activities of GLP were estimated according to the procedure described by Ye et al., with some minor modifications. After 30 min incubation at 25°C in
the dark, the mixture was shaken and absorbance at $\lambda=517$ nm was determined.

$$\text{Scavenging activity (\%) = \left[1 - \frac{A_2 - A_0}{A_1 - A_0}\right] \times 100\%}$$ (2)

Where $A_1$ corresponds to the presence of DPPH• and GLP, $A_0$ corresponds to the presence of DPPH• without GLP, and $A_2$ corresponds to the presence of GLP without DPPH•. The inhibition curves were prepared and IC$_{50}$ value was recorded.

### Table 2. The orthogonal test results.

| No. | A (°C) | B (h) | C | D (mL/g) | GLP Yield (mg/g) |
|-----|--------|-------|---|----------|------------------|
| 1   | 90     | 3.5   | 2.5| 15       | 63.72            |
| 2   | 90     | 4.0   | 3.0| 20       | 68.34            |
| 3   | 90     | 4.5   | 3.5| 25       | 61.38            |
| 4   | 95     | 3.5   | 3.0| 25       | 68.71            |
| 5   | 95     | 4.0   | 3.5| 15       | 71.46            |
| 6   | 95     | 4.5   | 2.5| 20       | 73.16            |
| 7   | 100    | 3.5   | 3.5| 20       | 72.66            |
| 8   | 100    | 4.0   | 2.5| 25       | 78.32            |
| 9   | 100    | 4.5   | 3.0| 15       | 75.33            |
| $K_1$ | 64.43 | 68.33 | 71.67 | 70.13 |
| $K_2$ | 71.07 | 72.63 | 70.77 | 71.33 |
| $K_3$ | 75.37 | 69.90 | 68.43 | 69.40 |
| $R$ | 1.094 | 0.430 | 0.324 | 0.193 |

#### 2.5.2. Hydroxyl radical (HO•) scavenging assay

HO• scavenging activity was tested according to a procedure available in the literature, with some minor modifications.

$$\text{Scavenging activity (\%) = \left[1 - \frac{A_2 - A_0}{A_1 - A_0}\right] \times 100\%}$$ (3)

Where $A_1$ corresponds to the presence of the test sample or Vc, $A_0$ corresponds to the presence of the deionized water, and $A_2$ corresponds to the presence of the reagent blank without sodium salicylate. The inhibition curves were prepared and IC$_{50}$ values were obtained.

#### 2.6. Statistical analysis

Each adherence assay was conducted, at least, in triplicate. The results were given error bars as means standard deviation obtained from three measurements, and values of $P \leq 0.05$ were considered significant. The standard deviation and mean values were calculated using the software Microsoft Excel 2010.

#### 3. Results and discussion

##### 3.1. Effect of EOW pH on GLP yield

EOW pH is an important factor for GLP extraction. While water-to-solid ratio, temperature and extraction time were 20 mL/g, 100°C, and 4 h, respectively. The lowest pH of 2.5 was used due to the limitation can be achieved in EOW.

Figure 1a shows that, the GLP yield increased with decrease in pH, and reached the maximum at pH 2.5. This may be explained by the stronger oxidizing capability and higher reactivity of low EOW pH compared to that of deionized water.
3.2. Effect of extraction temperature GLP yield

Effect of temperature (60-100°C) on GLP yield was studied, with other parameters being: EOW pH 2.5, water-to-solid ratio 20 mg/L and extraction time 4 h. Figure 1b shows that GLP yields increased significantly from 60 to 80°C, and slowed down when the temperature increased from 80 to 100°C. The positive reasons for this phenomenon might due to the higher diffusivities and improved mass transfer of the extracted molecules at higher temperature.

3.3. Effect of extraction time on GLP yield

Extraction time is another important factor influencing the polysaccharides extraction. Extraction time was set at 2, 3, 4, 5 and 6 h, respectively, with other parameters being: EOW pH 2.5, water-to-solid ratio 20 mL/g and extraction temperature 100°C. Fig. 1c shows that the GLP yield increased quickly within the initial 4 h, and reached the maximum value at 6 h.

3.4. Effect of water-to-solid ratio on GLP yield

Many studies have shown that different water-to-solid ratios may significantly affect the yield of polysaccharides. Water-to-solid ratio was set at 10, 20, 30, 40, and 50 mL/g, respectively. With other parameters being: EOW pH 2.5, temperature 100°C and extraction time 4 h. Fig. 1d shows that GLP yield increased remarkably as the water-to-solid ratio was increased from 10 to 30 mL/g, while the increase slowed down above a ratio of 30 mL/g. The results could be explained based on the faster diffusion of polysaccharides at a higher water-to-solid ratio. After 30 mL/g, the curve slightly levelled off, implying that further increase of ratio could not increase the GLP yield. This observation may be attributed to increased wastage of polysaccharides caused by larger volume of water during the procedure.

3.5. Optimization of GLP extraction conditions

The interaction effects among the variables does not take into consideration in single variable optimization, thus it may not reflect the net effects of the various parameters on the reaction rate. The maximum extraction yield of 78.47 ± 2.95 mg/g was achieved with parameters being: EOW pH 2.5, water-to-solid ratio 20 mg/L, temperature 100°C and extraction time 4 h. Till date, low extraction yield and high purification cost are the main reasons limited the commercialization of fungal polysaccharides. Our results indicated that the GLP yield was highly increased by EOW extraction.
3.6. Antioxidant activities of GLP
Strong antioxidant properties have been identified for GLPs, which make them radio-protective, tumor preventive, etc. Moreover, GLP was able to decrease the oxygen-free radicals production and playing an important role in against ageing.

3.6.1. DPPH• scavenging activity. DPPH• ethanol solution has a characteristic absorption maximum at \( \lambda = 517 \) nm, and the absorption could be expressed after accept an electron or hydrogen radical. Therefore, the ability of scavenging DPPH• can express the sample’s antioxidant activity.

Figure 2ashows that the scavenging activity of GLP on DPPH• was obvious and concentration-dependent. Furthermore, its ability of scavenging DPPH• increased significantly at relatively low concentration (0.2-1.0 mg/mL), and the scavenging activities of GLP was 78.93% at 1.0 mg/mL.

3.6.2. HO• scavenging activity. Figure 2bshows the scavenging activities of GLP on HO•. With the sample concentration increased, the scavenging effects of GLP increased, which indicate the dose-response of the scavenging effect. When the concentrations of GLP and Vc were 1.0 mg/mL, the HO• scavenging activities were 61.76% and 94.45% respectively. This result proved that GLP had HO• scavenging activity, although it was weaker than that of Vc (P<0.05). The effect of HO• scavenging activities may be enabled by the hydrogen supply of GLP, which combines with HO• and terminate the radical chain reaction.

![a] DPPH• scavenging activity

![b] HO• scavenging activity

Fig. 2 Antioxidant activities of GLP and Vc.

4. Conclusion
In this study, we established an efficient EOW extraction process for achieving high yield of GLP. The GLP yield under the optimal extraction condition (extraction time, temperature, pH and water-solid ratio were 4 h, 100°C, pH 2.5 and 20 mL/g, respectively) was 78.47 ± 2.95 mg/g. Furthermore, the antioxidant activities of GLP was evaluated in vitro by DPPH• and HO• scavenging assays.

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