Purification, composition analysis and antioxidant activity of a polysaccharide from the fruiting bodies of *Ganoderma atrum*

Yi Chen, Ming-Yong Xie *, Shao-Ping Nie, Chang Li, Yuan-Xing Wang

State Key Laboratory of Food Science and Technology, Nanchang University, Nanchang 330047, China

Abstract

A water-soluble protein-bound polysaccharide was extracted from the fruiting bodies of *Ganoderma atrum* and isolated by gel-filtration chromatography. Its primary structural features and molecular weight were characterized by infrared spectrometry, gas chromatography, size exclusion chromatography, amino acid analyzer and high-performance liquid chromatography (HPLC). The data obtained indicated that the glycoprotein contains 10.1% of protein and 17 general amino acids and it is rich in glutamic acid, asparagic acid, alanine, glycine, threonine, and serine. It was mainly composed of mannose, galactose and glucose in a molar ratio of 1:1.28:4.91, with an average molecular weight of about 1013 kDa. The existence of an *O*-glycosidic linkage in PSG-1 (polysaccharide1) was demonstrated by a β-elimination reaction. The antioxidant activity of the purified polysaccharides was evaluated *in vitro* by 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay, self-oxidation of 1,2,3-phentriol assay. Those various antioxidant activities were compared to standard antioxidants vitamin C and BHT. It was found that the scavenging effects of the purified polysaccharides increased with measuring concentration. The results indicated that the purified polysaccharides showed strong DPPH free radical and superoxide anion radical scavenging activities. This study suggested that the purified polysaccharides could potentially be used as natural antioxidants.
© 2007 Elsevier Ltd. All rights reserved.

Keywords: *Ganoderma atrum*; Glycoprotein; Heteroglycan; Structure elucidation; Antioxidant activity

1. Introduction

The genus *Ganoderma* is one of the most valued Chinese traditional medicines. It is well known as "Lingzhi" in Chinese, "Reishi" in Japanese, and "Youngzhi" in Korean. The fruiting bodies, cultured mycelia, and spores of them were reported to be effective in the treatment of chronic hepatopathy, hypertension, hyperglycemia and neophasia (Bao & Wang, 2002; Franz, 1989; Furusawa, Chou, Furusawa, Hirazami, & Dang, 1992; Liu, Shimizu, & Konishi, 2007; Shiao, Lee, Lin, & Wang, 1994). This fungus has attracted considerable attention because its polysaccharides have been demonstrated by recent research to possess diverse and potentially significant pharmacological activities such as anti-tumor, anti-aging (Miyazaki & Nishijima, 1981; Wang et al., 1997), hypoglycemic (Hikino, Konno, Mirin, & Hayashi, 1985; Tomoda, Gonda, Kasahara, & Hikino, 1986) and anti-microbial/viral activities (Eo, Kim, Lee, & Han, 2000; Yoon, Eo, Kim, Lee, & Han, 1994), including anti-human immunodeficiency virus (HIV) (El-Mekkawy et al., 1998; Kim, Shim, Choi, & Kim, 1997) activities.

In general, only two members of the genes *Ganoderma* were commonly known to possess medicinal/nutritional values, which were *Ganoderma lucidum* (red) and *Ganoderma tsugae* (red-brown). Up to now, most of the previous studies were just concentrated on these two members. It had been reported that three anti-tumor heteropolysaccharide–protein complexes with the molecular mass between 1.0 and 1.6×10^4 Da had been isolated from the mycelium of *G. tsugae* (Zhang et al., 1994). Chen, Zhang, Yu, and Zhu (2000) reported that a β-D-glucanprotein complex with

* Corresponding author. Fax: +86 791 3969069.
  E-mail address: myxie@ncu.edu.cn (M.-Y. Xie).

molecular mass of 64.6×10^4 Da was extracted from the *G. lucidum* mycelium. Bao, Liu, Fang, and Li (2001) isolated a polysaccharide with a molecular weight of 1.26×10^5 Da from the sporoderm-broken spores of *G. lucidum* and found that the polysaccharide showed a strong antibody and lymphocyte suppressive activity. In 2005, Peng and Zhang (2005) obtained six branched $(1 \rightarrow 3)$-β-D-glucans and $(1 \rightarrow 4)$-α-D-glucans water-soluble polysaccharides with high anti-tumor activity from the crude extra-cellular polysaccharide of *G. tsugae* mycelium. In addition to its therapeutic effects, the methanolic and water extracts from "Lingzhi" including *G. lucidum* and *G. tsugae* were found high in antioxidant abilities (Gong, Xie, & Chen, 2006; Mau & Tsai, 2005; Mau, Lin, & Chen, 2002; Mau, Tsai, Tseng, & Huang, 2002; Yang, Lin, & Mau, 2002; Yen & Wu, 1999). However, nowadays more and more studies revealed that another member – *Ganoderma atrum* – could also be used to promote health and longevity (Gao, Chan, & Zhou, 2004; Gao et al., 2005). However, the structures and antioxidant activity of the polysaccharides presenting in *G. atrum* have not been fully characterized yet. Besides, the wild mushroom of *G. atrum* is scarce.

In this work, one novel water-insoluble polysaccharide was extracted and purified from the fruiting bodies of cultivated *G. atrum*. Its chemical structures were characterized, and its antioxidant activities were reported for the first time. The result of this study introduced *G. atrum* as a possible valuable source for β-D-heteroglycan which helped to exhibit unique antioxidant properties.

2. Materials and methods

2.1. Materials

The fruiting bodies of *G. aturm* were cultivated and collected in Ganzhou, Jiangxi Province, China. This work was carried out with the dried fruiting bodies of *G. aturm*, which were cut into smaller pieces and further ground into powder by a mill. After several repetitions of milling, practically all the mycelia were broken into multiple pieces.

Sodium hydroxide (50%), inositol, hydroxylamine hydrochloride, acetic anhydride, pyridine, trifluoroacetic acid (TFA), methanol, and acetic acid were from Shanghai Chemicals and Reagents Co. (Shanghai, China). The standard monosaccharides (D-glucose, D-xylose, D-galactose, D-ribose, L-arabinose, D-glucuronic acid) were purchased from Merck Co. (Darmstadt, Germany) and Sigma Chemical Co. (St. Louis, MO, USA). Dextrans of different molecular weights were from Pharmacia Co. (Uppsala, Sweden). Hiload 26/60 Superdex-200 prep grade was from the Pharmacia Co. (Sweden). All other reagents used were of analytical grade.

2.2. General methods

UV–Vis absorption spectra were recorded with a TU-1901 UV–Vis Double Beam Spectrophotometer (PGEN-ERAL, Beijing, China). The FT-IR spectra (KBr pellets) were recorded on a Nicolet 5700 FT-IR spectrophotometer (ThermoElectron, Madison, WI, US). Total carbohydrate content was determined by the phenol–sulfuric acid method as D-glucose equivalents (Dubois, Gilles, Hamilton, & Smith, 1956). Proteins were detected by the Lowry method with BSA as a reference protein (Lowry, Rosebrough, Farr, & Randall, 1951).

GPC was performed with a Waters instrument, using Waters 2410 RI and 2996 DVD detector (Waters, USA).

Gas chromatography (GC) of the alditol acetate derivatives of monosaccharides was done on an Agilent 6890 GC (Agilent Technologies, USA) with a capillary column HP-5 (30 m × 0.32 mm i.d., film thickness 0.25 mm) and a flame-ionization detector and at temperatures programmed from 160 to 240 °C at 5 °C/min.

2.3. Isolation and purification of the polysaccharide

Standard procedure was followed for the isolation of the polysaccharide. The dried fruiting bodies of *G. atrum* were ground into fine particles (100 g) and defatted with 95% ethanol at room temperature for 48 h under stirring, then extracted with 2 l of double-distilled water for 2 h at 100 °C and filtered. The residue was further extracted with 750 ml of water for 1 h. The combined aqueous extracts were concentrated in a rotary evaporator under reduced pressure at 50 °C and filtered. Then the filtrate was precipitated by adding ethanol (4 times the volume of aqueous extract) at 4 °C, followed by centrifugation at 4800 rpm for 20 min. The precipitate was dissolved in 300 ml of water and deproteinized 20 times with 60 ml of 5:1 CHCl$_3$–*n*-BuOH as described by Sevag (Staub, 1965). The resulting aqueous fraction was extensively dialyzed against double-distilled water for 3 days and precipitated again by adding fourfold volume of ethanol. After centrifugation, the precipitate was washed with anhydrous EtOH and then dissolved in water and lyophilized to yield the crude polysaccharide (3.1 g) corresponding to polysaccharide of *G. atrum* (PSG) in the subsequent description.

The crude polysaccharide PSG (500 mg) was dissolved in distilled water, applied to a Hiload 26/60 Superdex-200 (2.6 × 60 cm) with the AKTA Purifier system (Amersham Pharmacia Biotech, Sweden), and eluted with water. Each fraction of 8 ml was collected at a flow rate of 120 ml/h and monitored by the phenol–sulfuric acid method at 490 nm. The elution profile detected by the phenol–sulfuric acid assay showed two big overlapping elution peaks namely as PSG-1 and PSG-2, respectively. Fractions of PSG-1 were collected and combined. After concentration, the collected PSG-1 fractions were dialyzed and lyophilized. To test the homogeneity of the purified polysaccharide, gel filtration chromatography on HPLC with a Ultrahydrogel-500 column was used. The product (50 mg) was subjected to the subsequent analyses.

2.4. Chemical analysis

2.4.1. Gel-permeation chromatography (GPC) analysis

The homogeneity and molecular weight of PSG-1 was determined on a Waters HPLC system (UK6 injector and 515 HPLC pump, Waters, Milford, MA) equipped with a Waters Ultrahydrogel-500 column (7.8 × 300 mm) and a Waters 410 differential refractometer. A sample solution (20 μl of 0.1% polysaccharide) was injected in each run, with 0.1 mol/l NaCl as the mobile phase at 0.6 ml/min.

The molecular weight of the purified polysaccharide was determined by a gel chromatography technique. Standard dextrans T-2000, T-500, T-150, T-100, T-70, T-40, T-10 and glucose were passed through the column, and then the elution volumes were plotted against the logarithm of their respective molecular weights. The elution volume of the purified polysaccharide was plotted in the same graph, and the molecular weight was determined.

2.4.2. Monosaccharide analysis

The polysaccharide PSG-1 (20 mg) was hydrolyzed separately with 2 M CF$_3$COOH (14 ml) for 6 h at 100 °C in a sealed glass tube. The excess acid was completely removed at 70 °C by a steady stream of nitrogen, and then the hydrolyzed products were prepared for acetylation. The acetylation was carried out with 10 mg hydroxylamine hydrochloride and 0.5 ml pyridine by heating in a water bath for 30 min at 90 °C. After incubation, the tubes were removed from the heat block, allowed to cool to room temperature, and then 0.5 ml of acetic anhydride was added and mixed thoroughly by vortexing. The tubes were sealed and incubated in a water bath shaker set at 90 °C for 30 min again. After cooling, approximately 0.1 ml of clear supernatant was added to the autosampler vials with inserts for injection into the gas chromatograph. Alditol acetates of authentic standards (D-glucose, D-mannose, D-galactose, D-fucose and D-arabinose) with myo-inositol as the internal standards were prepared and subjected to GLC analysis separately in the same way. The following chromatographic conditions were used: high-purity helium was used as the carrier gas at a flow rate of 1.2 ml/min. The temperature of the injector and detector was 240 °C. An initial column temperature held at 160 °C for 2 min, then programmed at a rate of 5 °C/min to 240 °C and held at 240 °C for a 10 min bakeout. Injections were made in the splitless mode.

2.4.3. Analysis of protein, uronic acid contents and amino acid composition

Proteins were estimated by colourimetric assay (Lowry et al., 1951), using bovine serum albumin as the standard. Amino acids were released by hydrolysis in vacuum with 6 M HCl at 110 °C for 22 h in a sealed tube, and were analyzed as described (Mazumder, Morvan, Thakur, & Ray, 2004). The amino acid composition was determined using a Hitachi 835-50G automatic amino acid analyzer (Hitachi Ltd., Tokyo, Japan).

Uronic acid contents were determined by measuring the absorbance at 525 nm using the *m*-hydroxybiphenyl colourimetric procedure and with D-glucuronic acid as the standard (Blumenkrantz & Asboe-Hansen, 1973).

2.4.4. Infrared spectroscopy

Infrared spectra of polysaccharides were recorded on a Thermo Nicolet 5700 infrared spectrophotometer. Samples were dried at 35–44 °C in vacuum over P$_2$O$_5$ for 48 h prior to making pellet with KBr powder.

2.4.5. Analysis of glycan–peptide bond

The linkage of PSG-1 was analyzed by the β-elimination reaction (Elizabeth, 1998; Zhu & Zhou, 2005). PSG-1 (10 mg/ml) was incubated with 0.2 mol/l NaOH containing 1.0 mol/l NaBH$_4$ for 16 h at 45 °C, scanned from 200 nm to 260 nm by UV–Vis spectrophotometer, and then was compared with the sample without alkali treatment.

2.5. Assay for antioxidant activities

2.5.1. Effect of scavenging 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radicals

The free radical scavenging activity of the purified polysaccharides was measured by 1,1-diphenyl-2-picryl-hydrazyl (DPPH) test according to the method of Shimada, Fujikawa, Yahara, and Nakamura (1992), with some modifications. The 0.2 mmol/l solution of DPPH in 95% ethanol was prepared daily before UV measurements. One milliliter of the purified polysaccharides of different addition quantity (0.125–4 mg) in water was thoroughly mixed with 2 ml of freshly prepared DPPH and 2 ml of 95% ethanol. The mixture was shaken vigorously and left to stand for 30 min in the dark, and the absorbance was then measured at 517 nm against a blank. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity, which was analyzed from the graph plotted of inhibition percentage against compound concentration. Ascorbic acid and BHA were used as positive controls. The experiment was carried out in triplicate and averaged.

The capability to scavenge the DPPH radical was calculated using the following equation:

$$I\% = [1 - (A_i - A_j)/A_c] \times 100\%$$

where A_c is the absorbance of DPPH solution without sample (2 ml DPPH + 3 ml of 95% ethanol); A_i is the absorbance of the test sample mixed with DPPH solution (1 ml sample + 2 ml DPPH + 2 ml of 95% ethanol) and A_j is the absorbance of the sample without DPPH solution (1 ml sample + 4 ml of 95% ethanol).

2.5.2. Self-oxidation of 1,2,3-phentriol assay

The scavenging abilities for self-oxidation of 1,2,3-phentriol of all different contents were investigated according to the method of Marklund and Marklund (1974) with a minor modification. Briefly, samples were dissolved in distilled water at 0 (control), 0.5, 1, 2 and 4 mg/ml. The

sample solution (0.1 ml) was mixed with 5 ml of 0.05 M Tris–HCl buffer (pH 8.2) and incubated at 25 °C in a water bath for 10 min. Then 1,2,3-phentriol (0.2 ml, 6 mM) was added, and the mixture was shaken rapidly at room temperature. The absorbance of the mixture was measured at 325 nm per 30 s for 4 min against a blank, and a slope was calculated as absorbance/min. The ability of different scavenging ability for self-oxidation of 1,2,3-phentriol of all fractions was calculated using the equation:

$$\text{Scavenging effect}(\%) = (1 - \text{slope of sample/slope of control}) \times 100\%.$$

The increase in the absorbance of the reaction mixture indicated an increase in the restraining power. Ascorbic acid and BHT were used as positive controls.

3. Results and discussion

3.1. Isolation of PSG-1

The parent polysaccharide, named PSG, was obtained as a water-insoluble dust-coloured powder from the fruiting bodies of *G. atrum* by hot water extraction. The total yield of water-soluble polysaccharides was 3.9% by this isolation procedure, higher than the yield of 1.8% from *G. tsugae* mycelium extracted by the same procedure (Zhang et al., 1994) and almost equal to the yield of 3.8% extracted from *G. lucidum* fruiting bodies with the similar process (Bao & Wang, 2002). This yield of 3.9% was also much higher than the yield of 0.15% from the fruiting bodies of *G. lucidum* extracted by sodium phosphate buffer (Zhang & Chen, 1997) and the yield of 0.82% from the fruiting bodies of *G. lucidum* extracted by chloroform followed by hot water (Han, Nakamura, & Hattori, 2006). Therefore, the adopted conditions of the isolation procedure here were helpful to obtain water-soluble polysaccharides from the *G. atrum* fruiting bodies in a greater yield. The crude polysaccharide was separated and sequentially purified through a Hiload 26/60 Superdex-200 column, giving two big overlapping elution peaks: PSG-1 and PSG-2 (eluted with water) (Fig. 1), as detected by the phenol–sulfuric acid assay. And Fig. 2 shows the chromatogram of the polysaccharides detected by AKTA Purifier system. One contains the high molecular weight polysaccharide, and the second mostly contains the low molecular weight polymer. PSG-2 was more complex and not further investigated here. PSG-1 was collected for further identification of structure and monosaccharide compositions.

3.2. Chemical analysis of PSG-1

3.2.1. Gel-permeation chromatography (GPC) analysis

PSG-1 was further eluted as a single and symmetrically sharp peak from gel-permeation chromatography on Ultrahydrogel-500 column with HPLC (Fig. 3), in which the protein and the sugar peak appeared at the same time,
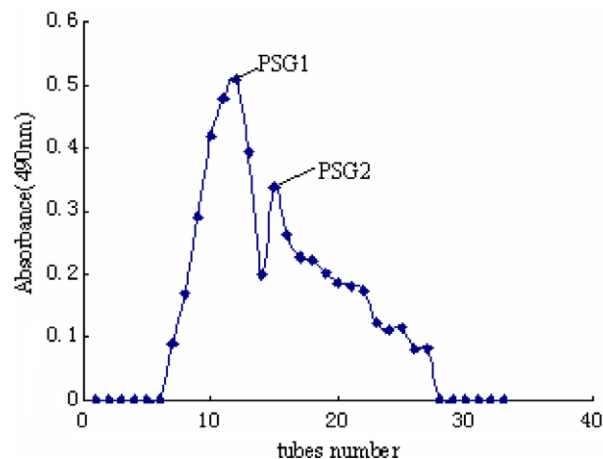

Fig. 1. Superdex-200 column elution profile of the polysaccharides present in the aqueous extract of *Ganoderma atrum*. Eluent: H_2O, flow rate: 2 ml/min, 8 ml/tube.

indicating that PSG-1 was a homogeneous protein-bound polysaccharide, with a weight-average molecular weight based on column calibration of 1013 kDa. The calibration curve prepared with standard dextrans was shown in Fig. 4. It had a positive response to the Lowry test and absorption at 280 nm or 260 nm in the UV spectrum, indicating the existence of protein and nucleic acid. The yields, average molecular weight, protein content, uronic acid contents, and sugar compositions of PSG-1 were determined and given in Table 1. The total sugar content of the polysaccharide was determined to be 89.1% with a purity of >99.8% in PSG-1, using the phenol–sulfuric acid method. Protein was estimated by Lowry method. Though the protein content of PSG-1 was a little high, it could be considered to be protein-bound polysaccharide because the Sevag method was repeated many times to remove free proteins. Generally, the pure protein exists as a globular shape in aqueous solution, but protein-bound polysaccharides exhibit a relatively expanded flexible chain, such as proteoglycan monomers (Ghosh & Reed, 1995). Therefore, we can presume that PSG-1 exhibits a more expanded flexible coil rather than a compact coil chain.

3.2.2. Monosaccharide composition

The HPLC results indicated that the PSG-1 mainly consisted of D-glucose. On hydrolysis by 2 M TFA, the presence of D-glucose, D-mannose, and D-galactose was detected by GLC analysis (Fig. 5). Usually, GC analysis could give the accurate content of sugars in the polysaccharides. The experimental results from GC are summarized in Table 1. The predominant monosaccharide in PSG-1 samples was D-glucose, which was consistent with the results of HPLC. These sugars (mannose, galactose and glucose) were found to be present in a molar ratio of 1:1.28:4.91. The absolute configuration of the monosaccharide was determined by GLC examination of acetylated (+)-2-octyl glycosides and showed that all have D configurations (Gerwig, Kamerling, & Vliegenthart, 1979). The sugar
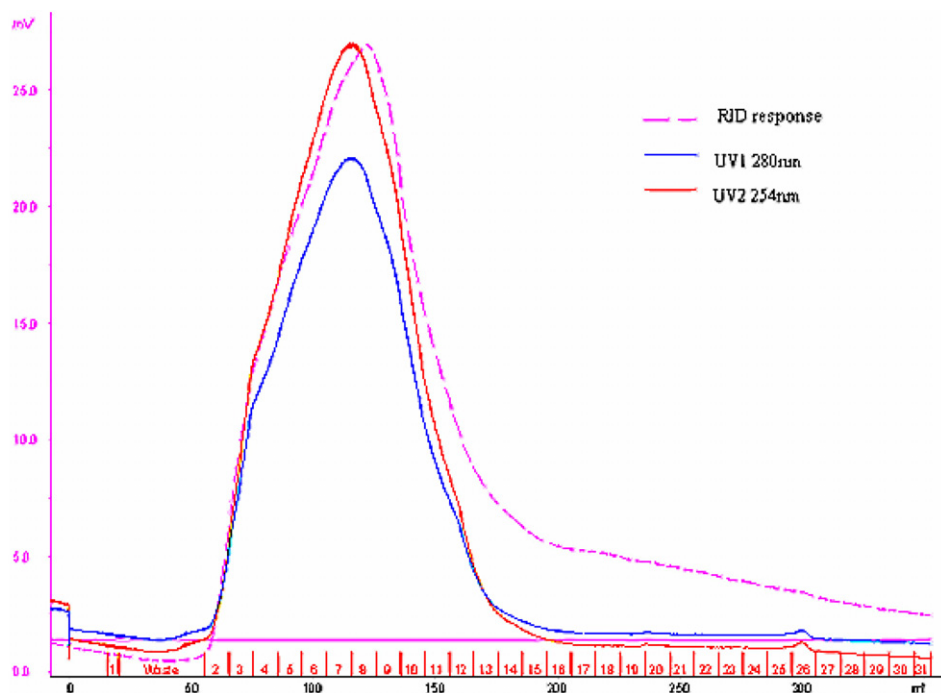
Fig. 2. The chromatography of *Ganoderma atrum* crude polysaccharides on Superdex-200 column detected by AKTA Purifier system.
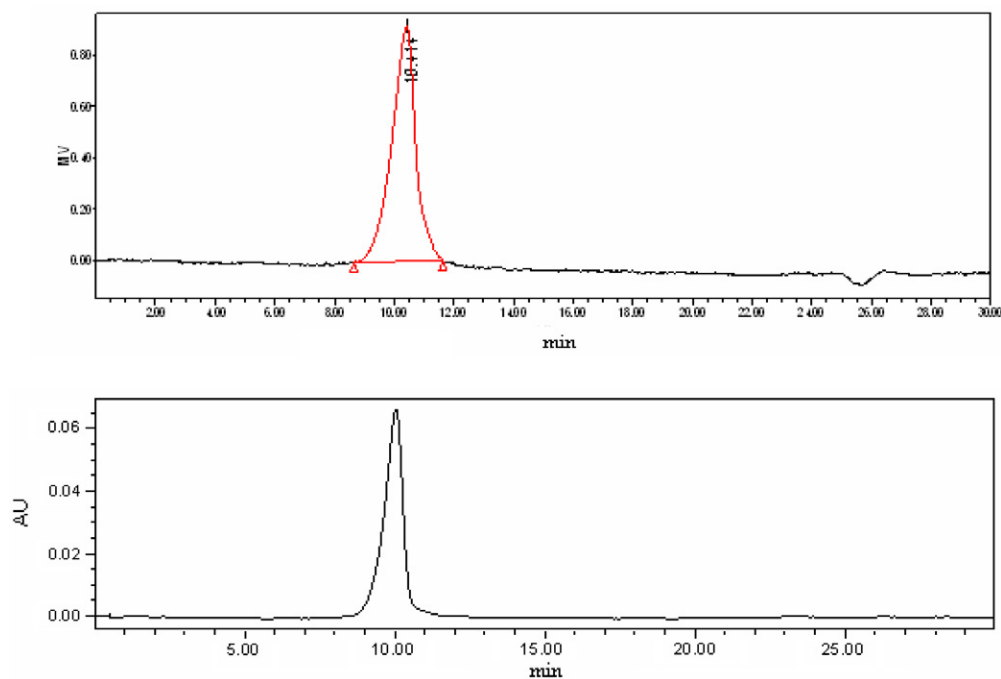

Fig. 3. Profile of PSG-1 in HPGPC on Ultrahydrogel-500 column, with 0.1 mol/l NaCl at 0.6 ml/min.

composition of this fraction (PSG-1) is similar to glycosyl residue compositions of PL-1 isolated from *G. lucidum* by Bao and Wang (2002), except that the latter is composed of Rha, Gal and Glc in the molar ratios of 1:4:13.

3.2.3. Amino acid composition and uronic acid content

The amino acid composition of PSG-1 was analyzed (Table 2). The glycoprotein contained 17 general amino

acids (Fig. 6) and was rich in glutamic acid, asparagic acid, alanine, glycine, threonine and serine. The high concentration of threonine and serine indicated the possibility of the existence of the *O*-glycosidic linkages.

3.2.4. IR spectroscopy

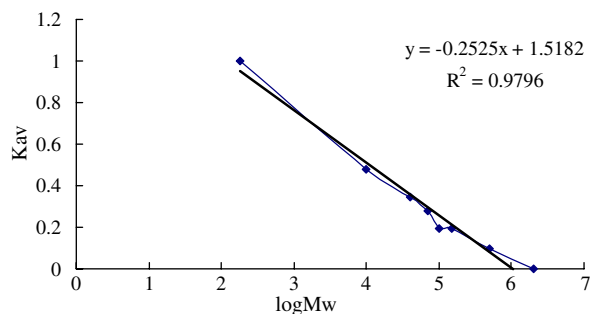The spectra were recorded at the absorbance mode from 4000 to 400 cm^{-1} (mid infrared region) at a resolution of

Fig. 4. Plot of $\log M_w$ of standard dextrans on GPC against their K_{av}.

844 cm^{-1}, while that of the C–H bond is in β-style nearby 891 cm^{-1} (Barker, Bourne, & Stacey, 1954). Fig. 7 shows the IR spectra of polysaccharide fractions (PSG-1) of *G. atrum*. The FT-IR spectrum of PSG-1 showed a strong band between 950 and 1160 cm^{-1} attributed to the stretching vibrations of pyranose ring. In the anomeric region ($950–700 \text{ cm}^{-1}$), the fraction exhibited the obvious characteristic absorption at 920 and 809 cm^{-1} corresponding to the existence of mannose (Mathlouthi & Koenig, 1986). A characteristic absorption at 899 cm^{-1} was also observed, indicating the β-configuration of the sugar units. There was no absorption at 850 cm^{-1} for the α-configuration.

The strong absorption at 1650 and 1548 cm^{-1}, corresponding to the stretching vibration of the carbonyl bond of the amide group and the bending vibration of the N–H bond, respectively, showed the existence of protein. Besides, absorption band over 1700 cm^{-1} indicated the

4 cm^{-1} with 128 co-added scans. At least triplicate spectra were recorded for each sample.

There are two types of end carbon-glucoside bonds: α- and β-styles, which can be judged by IR. In IR spectra, the C–H bond in α-style has an absorption peak nearby

Table 1
Yields, protein contents, sugar contents, uronic acid contents and M_w of PSG-1

Sample	Appearance	Yield[a,b] (%)	Protein (%)	Uronic acid (%)	M_w (Da)	Sugar component (%)		
						D-Glucose	D-Mannose	D-Galactose
PSG-1	Dust-colour, fluffy	2.1 (0.265)	10.11 (0.168)	15.57 (0.111)	1,012,745 (4.583)	68.3 (0.819)	13.9 (0.612)	17.8 (0.445)

[a] Calculated as weight ratio of PSG-1/PSG.
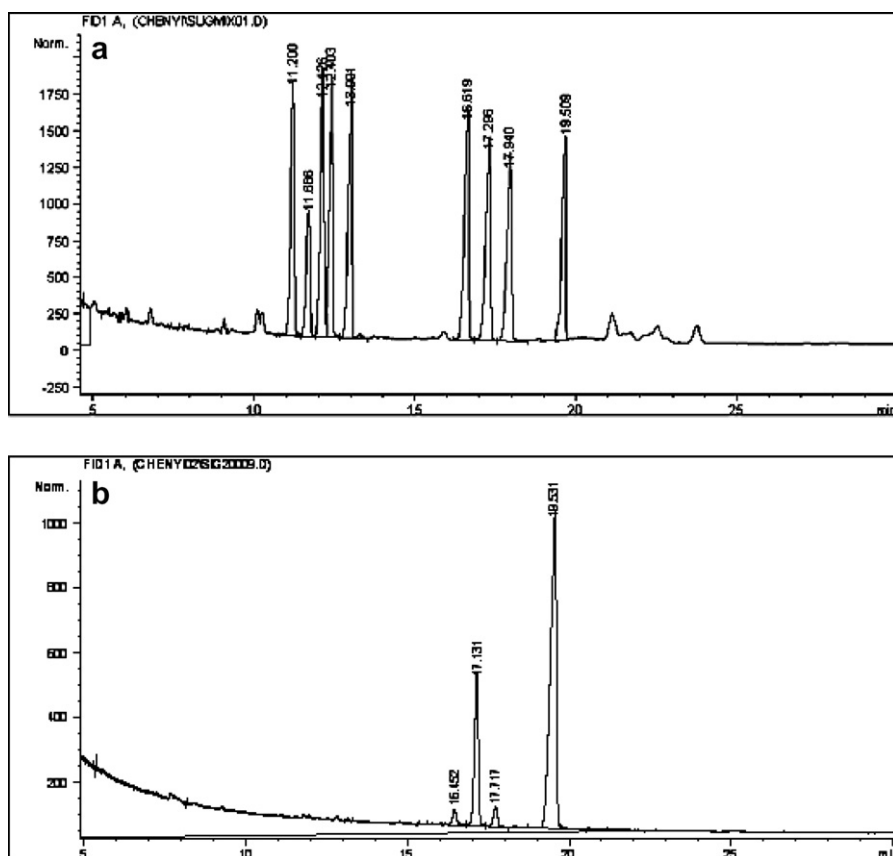[b] Data were shown as mean (SD), $n = 3$.

Fig. 5. Chromatograms of monosaccharides (as their acetylated aldononitriles): (a) standard mixture; and (b) sample. The last peak is the internal standard.

Table 2
The amino acid composition of PSG-1

Peak[a]	Name of amino acid	Retention time (min)	Area	Concentration (%)
1	Asp	4.77	141,779	0.691
2	Thr	5.55	160,457	0.674
3	Ser	6.23	186,334	0.662
4	Glu	7.03	158,512	0.858
5	Gly	10.51	187,606	0.513
6	Ala	11.72	168,857	0.638
7	Cys	12.57	6653	0.057
8	Val	13.16	77,713	0.358
9	Met	14.39	201,113	1.149
10	Ile	16.52	33,932	0.186
11	Leu	17.48	81,428	0.390
12	Tyr	18.07	16,090	0.101
13	Phe	18.84	37,273	0.195
14	Lys	21.11	68,379	0.281
16	His	23.48	3421	0.017
17	Arg	27.72	30,252	0.165
	Pro[b]	7.76	37,633	0.175
Total concentration of the 17 general amino acids				7.103
Proportion of the essential amino acids				45.52

[a] Refers to Fig. 6A1 and B1.
[b] Refers to Fig. 6A2 and B2.

trace of uronic acids in the samples. The absorption peak at ca. 1740 cm^{-1} was related to the free carboxyl group without coupling with benzylamine.

The bands in the region of 3428 cm^{-1} were due to the hydroxyl stretching vibration of the polysaccharides. The bands in the region of 2925 cm^{-1} were due to C–H stretch-

ing vibration. Absorptions at 915 cm^{-1} were typical for D-Glc in the pyranose form (Barker et al., 1954).

3.2.5. Linkage analysis

Linkages in the structure of glycoproteins can be divided into two types on the basis of their stability to alkali: *O*-glycosidic linkages and *N*-glycosidic linkages. The alkali-sensitive *O*-glycosidic linkages (involving Xyl, GlcNAc, Gal or Man and Ser or Thr) are easily split in relatively mild conditions by a β-elimination mechanism resulting in the release of the carbohydrate moiety. This method has widely been used to analyze the type of linkages in glycoproteins (Elizabeth, 1998). UV scanning spectra of the samples with and without alkali treatment are shown in Fig. 8. By comparison, the sample with alkali treatment had distinct higher absorbance at 241 nm than that without alkali treatment, showing that β-elimination reaction had taken place, which demonstrated that the protein and carbohydrate were linked by an O-linkage in PSG-1.

3.3. Antioxidant activity analysis

3.3.1. Effect of scavenging DPPH radicals

The model of scavenging the stable DPPH radical is a widely used method to evaluate the free radical scavenging ability of natural compounds (Lai, Chou, & Chao, 2001; Lee, Hwang, Ha, Jeong, & Kim, 2003; Leong & Shui, 2002; Nagai, Inoue, Inoue, & Suzuki, 2003). In the DPPH test, the antioxidants were able to reduce the stable DPPH
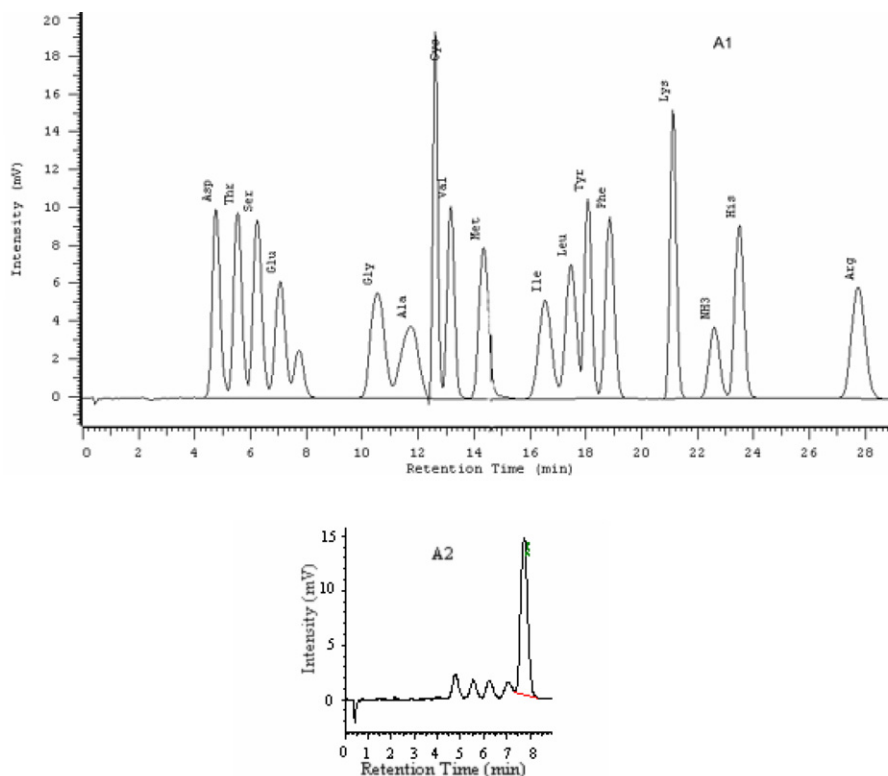

Fig. 6. The chromatogram of 17 kinds of amino acids: A1: 16 kinds of standard amino acids; A2: proline of standard; B1: 16 kinds of amino acids in sample; B2: proline of sample.
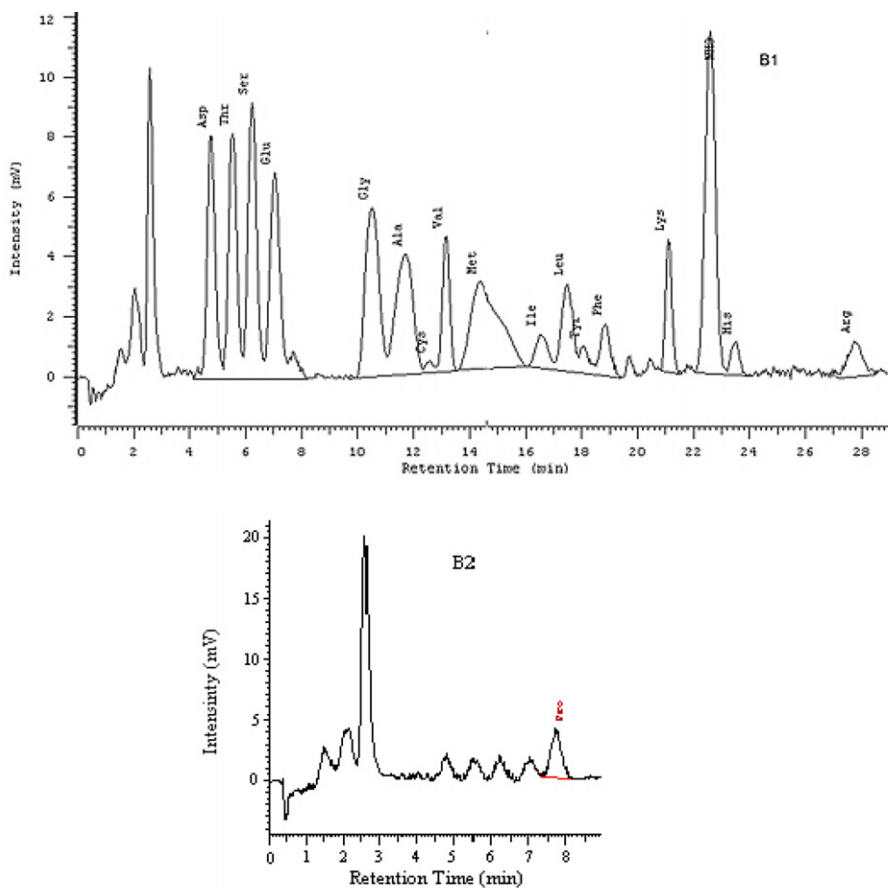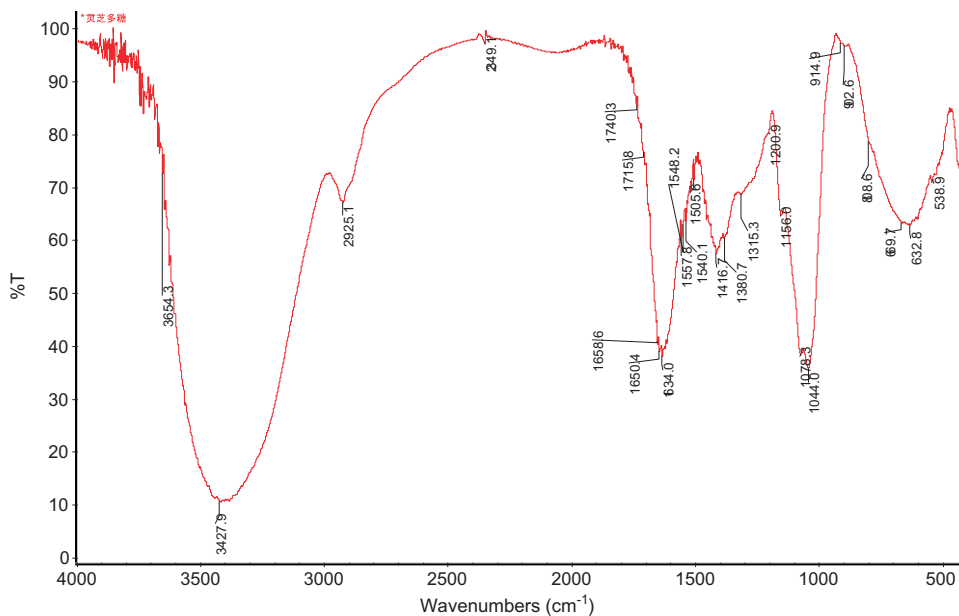
Fig. 6 (*continued*)

radical to the yellow-coloured diphenylpricrylhydrazine. The effect of antioxidants on DPPH radical scavenging was conceived to be due to their hydrogen-donating ability.

The scavenging ability of purified polysaccharides on hydrogen peroxide is shown in Fig. 9 and compared with vitamin C and BHT as control standards. Fig. 9 illustrates

Fig. 7. The IR spectra of polysaccharide fractions (PSG-1) of *Ganoderma atrum*.
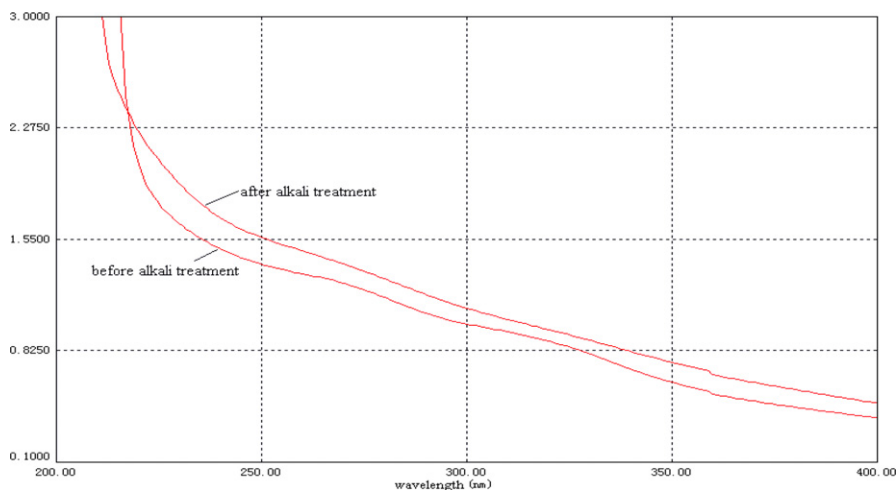
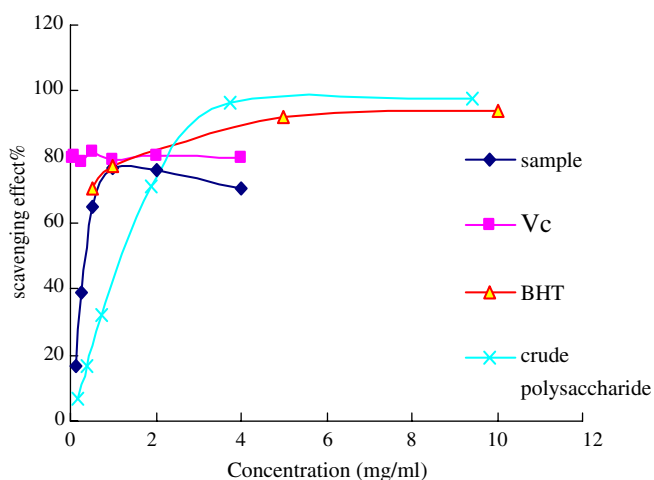Fig. 8. UV scanning spectrum of WGGP before and after alkali treatment.

Fig. 9. Scavenging effects of sample and control standards on DPPH free radicals; data are presented as mean value ($n = 3$).

which contributed to their antioxidant properties. Among these antioxidant components, there may be some interactions and synergistic effects for antioxidant properties. However, further investigation will be required to exactly explain why water decoction and crude PSG have better antioxidant effects than purified polysaccharide fraction (PSG-1).

3.3.2. Scavenging activity of self-oxidation of 1,2,3-phentriol by GM, GMA, GMB and GMC

Inhibition of pyrogallol auto-oxidation was performed according to the method of Marklund and Marklund (1974) with minor modifications. Superoxide anion radical can be generated by pyrogallol autooxidation and it can produce a coloured compound. Resulting from a colour change from purple to yellow, the absorbance at 320 nm increased when the superoxide anion was scavenged by an antioxidant, which can represent the content of superoxide radicals and indicate the antioxidant

that the scavenging effects of the purified polysaccharides, vitamin C and BHT on the DPPH radical concentration-dependently increased and were 76.9%, 79.3% and 77.2% at the dose of 1 mg/ml, respectively. Both of them presented approximately identical change trend of antioxidant activity. These results indicated that the purified polysaccharides have a noticeable effect on scavenging free radicals, especially at high addition quantity. However, the radical-scavenging activity of the purified polysaccharide was lower than that of vitamin C and BHA used in this study. Furthermore, the crude polysaccharides exhibited a relatively high level of radical-scavenging activity. The polysaccharide fraction (PSG-1) that was purified further by the gel-filtration column exhibited less antioxidant activity than the crude polysaccharides. The reason for this could be that the crude *G. atrum* extracts were rich in anti-oxidant components, such as proteins, amino acids, peptides, cellulose, phytosrterol, ascorbic acid, thiamine, nucleotide, nicotinic acid, organic acids and microelements,
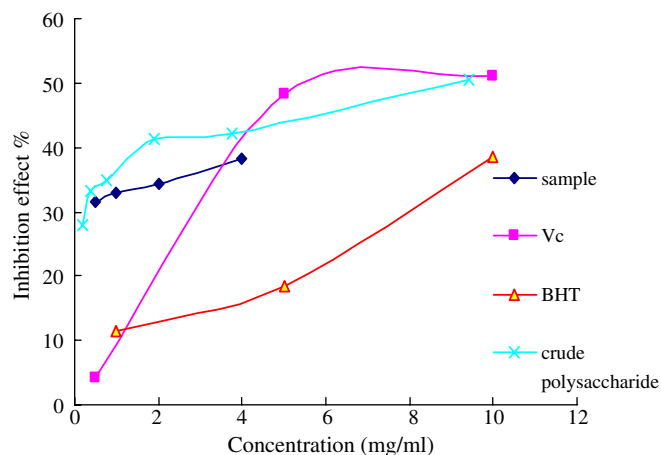

Fig. 10. Scavenging activity of self-oxidation of 1,2,3-phentriol by samples and control standards; data are presented as mean value ($n = 3$).

activity of the sample. Superoxide anion is one of the precursors of the singlet oxygen and hydroxyl radicals; it indirectly initiates lipid peroxidation. Apart from that, the presence of superoxide anion can magnify the cellular damage because it produces other kinds of free radicals and oxidizing agents.

Fig. 10 shows the scavenging power of self-oxidation of 1,2,3-phentriol of the polysaccharides extracted and purified from the fruiting bodies of *G. atrum*. The scavenging powers of samples and standards both correlated well with increasing concentrations, but that of samples was not as remarkable as that of standards. Below the dose of 4 mg/ml, the inhibitory effects of the crude and purified polysaccharides were markedly stronger than vitamin C, and after that, no longer obviously increased, whereas the inhibitory effect of vitamin C continued to increase and came up with the crude polysaccharides at the addition quantity of 10 mg/ml. Moreover, the scavenging power of crude polysaccharide was more pronounced than that of the purified polysaccharide, which is in accordance with the results of assay in Section 3.3.1. The scavenging effects of the extracts and standards decreased in the order of crude polysaccharide > purified polysaccharide > V_c > BHT at the addition of 2 mg/ml, respectively. These results indicated that PSG had a strong scavenging power for self-oxidation of 1,2,3-phentriol at low addition quantity and should be explored as novel potential antioxidants.

3.4. Discussion about the possible antioxidant mechanism of PSG-1

The natural anti-tumor polysaccharides isolated from mushroom include acidic and neutral ones with different types of glycosidic linkages, while some are bound to protein or peptide residues such as polysaccharide–protein or –peptide complexes (Cun et al., 1994; Jong, Birmingham, & Pai, 1991; Mizuno & Zhuang, 1995). Moreover, it has been reported that the superoxide anion radical scavenging activity of polysaccharide extracts appears to depend on the amount of peptides present in the form of polysaccharide–peptide complexes. For example, lentinan and schizophyllan, which contain only trace amounts of peptides in the polysaccharide samples, have almost no scavenging effect, whereas polysaccharide krestin and polysaccharopeptide, which are obtained from mushrooms, such as *G. lucidum* and *Grifola umbellate* which have lower polysaccharide/peptide ratios, exhibit the strongest scavenging effects (Behera, Verma, Sonone, & Makhija, 2005). The antioxidant mechanism may be due to the supply of hydrogen by PSG-1, which combines with radicals and forms a stable radical to terminate the radical chain reaction. The other possibility is that PSG-1 can combine with the radical ions which are necessary for radical chain reaction; then the reaction is terminated. However, the exact explanation of mechanism underlying the free radical scavenging activity exerted by polysaccharides is still not fully understood.

The future challenge is to define the 3D structure of polysaccharides and the structure–function relationship. This presents a good opportunity for scientists to elucidate the biological roles of polysaccharides and to design high potential anti-tumor drugs based on the 3D structures.

4. Conclusions

According to the results stated above, it could be concluded that the water extracted crude polysaccharide of *G. atrum* predominantly contained two polysaccharide fractions (PSG-1, PSG-2) after being purified by Hiload 26/60 Superdex-200 column chromatography, and the purified polysaccharide prepared (PSG-1) were confirmed of high purity. The present study also showed that PSG-1 consisted of a polysaccharide part and a protein part. PSG-1 was found to have strong antioxidant potential according to the *in vitro* evaluation of its free radical-scavenging and self-oxidation of 1,2,3-phentriol inhibitory activities, which may be comparable to vitamin C. We may rationally assume that *G. atrum* plays its curative effect in folk medicine partly as a result of the mechanism of antioxidation of polysaccharides in it, and so they should be explored as a novel potential antioxidant.

References

Bao, X. F., Liu, C. P., Fang, J. N., & Li, X. Y. (2001). Structural and immunological studies of a major polysaccharide from spores of *Ganoderma lucidum* (Fr.) Karst. *Carbohydrate Research, 332*(1), 67–74.

Bao, X. F., & Wang, X. S. (2002). Structural features of immunologically active polysaccharides from *Ganoderma lucidum*. *Phytochemistry, 59*(2), 175–181.

Barker, S. A., Bourne, E. J., Stacey, M., et al. (1954). Infrared Spectra of carbohydrates. Part I. Some derivatives of D-glucopyranose. *Journal of the Chemical Society, 1*, 171–176.

Behera, B. C., Verma, N., Sonone, A., & Makhija, U. (2005). Evaluation of antioxidant potential of the cultured mycobiont of a lichen *Usnea ghattensis*. *Phytotherapy Research, 19*(1), 58–64.

Blumenkrantz, N., & Asboe-Hansen, G. (1973). New method for quantitative determination of uronic acid. *Analytical Biochemistry, 54*(2), 484–489.

Chen, J., Zhang, L., Yu, D., & Zhu, R. (2000). The chemical structure and solution properties of polysaccharides from *Ganoderma lucidum* mycelium. *Chemical Journal of Chinese Universities, 21*(5), 961–964.

Cun, Z., Mizuno, T., Ito, H., Shimura, K., Sumiya, T., & Kawade, M. (1994). Antitumor activity and immunological property of polysaccharides from the mycelium of liquid-cultured *Grifola frondosa*. *Journal of the Japanese Society for Food Science and Technology, 41*, 724–733.

Dubois, M., Gilles, K. A., Hamilton, J. K., & Smith, F. (1956). Colourimetric method for determination of sugars and related substances. *Analytical Chemistry, 28*(3), 350–356.

Elizabeth, F. H. (1998). Analytical methods for the study of *O*-GlcNAc glycoproteins and glycopeptides. In *Glycoanalysis protocols* (pp. 19–29). Totowa, NJ: Humana Press.

El-Mekkawy, S., Meselhy, M. R., Nakamura, N., Tezuka, Y., Hattori, M., Kakiuchi, N., et al. (1998). Anti-HIV-1 and anti-HIV-1-protease substances from *Ganoderma lucidum*. *Phytochemistry, 49*(6), 1651–1657.

Eo, S. K., Kim, Y. S., Lee, C. K., & Han, S. S. (2000). Possible mode of antiviral activity of acidic protein bound polysaccharide isolated from *Ganoderma lucidum* on herpes simplex viruses. *Journal of Ethnopharmacology, 72*(3), 475–481.

Franz, G. (1989). Polysaccharides in pharmacy: Current applications and future concepts. *Planta Medica, 55*, 493–497.

Furusawa, E., Chou, S. C., Furusawa, S., Hirazami, A., & Dang, Y. (1992). Antitumor activity of *Ganoderma lucidum*: An edible mushroom, on intraperitoneally implanted Lewis Lung Carcinoma in synergenic mice. *Phytotherapy Research, 6*(2), 300–304.

Gao, Y. H., Chan, Eli, & Zhou, S. F. (2004). Immunomodulating activities of *Ganoderma*: A mushroom with medicinal properties. *Food Reviews International, 20*(2), 123–161.

Gao, Y. H., Tang, W. B., Gao, H., Chan, Eli, Lan, J., & Li, X. T. (2005). Antimicrobial activity of the medicinal mushroom *Ganoderma*. *Food Reviews International, 21*(2), 211–229.

Gerwig, G. J., Kamerling, J. P., & Vliegenthart, J. F. G. (1979). Determination of the absolute configuration of monosaccharides in complex carbohydrates by capillary G.L.C. *Carbohydrate Research, 77*, 1–7.

Ghosh, S., & Reed, W. F. (1995). New characteristic signatures from time-dependent static light scattering during polymer depolymerization, with application to proteoglycan subunit degradation. *Biopolymers, 35*(5), 435–450.

Gong, X. F., Xie, M. Y., & Chen, Y. (2006). Comparison of the anti-oxidative effects of extracts from *Ganoderma atrum* and *Ganoderma lucidum*. *Food Science (Chinese), 27*(4), 44–47.

Han, H. F., Nakamura, N., & Hattori, M. (2006). Protective effects of an acidic polysaccharide isolated from fruiting bodies of *Ganoderma lucidum* against murine hepatic injury induced by *Propionibacterium acnes* and lipopolysaccharide. *Journal of Natural Medicines, 60*(4), 295–302.

Hikino, H., Konno, C., Mirin, Y., & Hayashi, T. (1985). Isolation and hypoglycemic activity of *Ganoderma* A and B, glycan of *Ganoderma lucidum* fruiting bodies. *Planta Medica, 51*(4), 339–340.

Jong, S. C., Birmingham, J. M., & Pai, S. H. (1991). Immunomodulatory substances of fungal origin. *Journal of Immunological Immunopharmacology, 11*, 115–122.

Kim, H. W., Shim, M. J., Choi, E. C., & Kim, B. K. (1997). Inhibition of cytopathic effect of human immunodeficiency virus-1 by water-soluble extract of *Ganoderma lucidum*. *Archives of Pharmacal Research, 20*(5), 425–431.

Lai, L. S., Chou, S. T., & Chao, W. W. (2001). Studies on the antioxidative activities of hsian-tsao (*Masona procumbens Hemsl*) leaf gum. *Journal of Agricultural and Food Chemistry, 49*(2), 963–968.

Lee, S. E., Hwang, H. J., Ha, J. S., Jeong, H. S., & Kim, J. H. (2003). Screening of medicinal plant extracts for antioxidant activity. *Life Sciences, 73*(2), 167–179.

Leong, L. P., & Shui, G. (2002). An investigation of antioxidant capacity of fruits in Singapore markets. *Food Chemistry, 76*(1), 69–75.

Liu, J., Shimizu, K., & Konishi, F. (2007). Anti-androgenic activities of the triterpenoids fraction of *Ganoderma lucidum*. *Food Chemistry, 100*(4), 1691–1696.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin-Phenol reagents. *Journal of Biological Chemistry, 193*, 265–275.

Marklund, S., & Marklund, G. (1974). Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *European Journal of Biochemistry, 47*(3), 469–474.

Mathlouthi, M., & Koenig, J. (1986). Vibrational spectra of carbohydrate. *Advance in Carbohydrate Chemistry and Biochemistry, 44*, 7–89.

Mau, J. L., Lin, H. C., & Chen, C. C. (2002). Antioxidant properties of several medicinal mushrooms. *Journal of Agricultural and Food Chemistry, 50*(21), 6072–6077.

Mau, J. L., & Tsai, S. Y. (2005). Antioxidant properties of hot water extracts from *Ganoderma tsugae Murrill*. *LWT – Food Science and Technology, 38*(6), 589–597.

Mau, J. L., Tsai, S. Y., Tseng, Y. H., & Huang, S. J. (2002). Antioxidant properties of methanolic extracts from *Ganoderma tsugae*. In *Presented at the annual meeting of institute of food technologists*, Anaheim, CA, USA.

Mazumder, S., Morvan, C., Thakur, S., & Ray, B. (2004). Cell wall polysaccharides from chalkumra (*Benincasa hispida*) fruit. Part I. Isolation and characterization of pectins. *Journal of Agricultural and Food Chemistry, 52*(11), 3556–3562.

Miyazaki, T., & Nishijima, M. (1981). Structural examination of a water soluble, antitumor polysaccharide of *Ganoderma lucidum*. *Chemical and Pharmaceutical Bulletin, 29*(12), 3611–3616.

Mizuno, T., & Zhuang, C. (1995). *Grifola frondosa*: Pharmacological effects. *Food Reviews International, 11*(1), 135–149.

Nagai, T., Inoue, R., Inoue, H., & Suzuki, N. (2003). Preparation and antioxidant properties of water extract of propolis. *Food Chemistry, 80*(1), 29–33.

Peng, Y. F., & Zhang, L. (2005). Structure and antitumor activities of the water-soluble polysaccharides from *Ganoderma tsugae* mycelium. *Carbohydrate Polymers, 59*(3), 385–392.

Shiao, M. S., Lee, K. R., Lin, L. J., & Wang, C. T. (1994). Natural products and biological activities of the Chinese medical fungus, *Ganoderma lucidum*. In C. T. Ho, T. Osawa, M. T. Huang, & R. T. Rosen (Eds.), *Food phytochemicals for cancer prevention. Part II: Teas, spices and herbs* (pp. 342–354). Washington, DC: American Chemical Society Press.

Shimada, K., Fujikawa, K., Yahara, K., & Nakamura, T. (1992). Antioxidative properties of xanthone on the auto oxidation of soybean in cylcodextrin emulsion. *Journal of Agricultural and Food Chemistry, 40*(6), 945–948.

Staub, A. M. (1965). Removal of protein – Sevag method. *Methods in Carbohydrate Chemistry, 5*(2), 5–6.

Tomoda, M., Gonda, R., Kasahara, Y., & Hikino, H. (1986). Glycan structures of *Ganoderma lucidum* B and C, hypoglycemic glycans of *Ganoderma lucidum* fruiting bodies. *Phytochemistry, 25*(12), 2817–2820.

Wang, S. Y., Hsu, M. L., Hsu, H. C., Tzeng, C. H., Lee, S. S., Shiao, M. S., et al. (1997). The anti-tumor effect of *Ganoderma lucidum* is mediated by cytokines reduced from activated macrophages and T lymphocytes. *International Journal of Cancer, 70*(6), 699–705.

Yang, J. H., Lin, H. C., & Mau, J. L. (2002). Antioxidant properties of several commercial mushrooms. *Food Chemistry, 77*(2), 229–235.

Yen, G. C., & Wu, J. Y. (1999). Antioxidant and radical scavenging properties of extracts from *Ganoderma tsugae*. *Food Chemistry, 65*(3), 375–379.

Yoon, S. Y., Eo, S. K., Kim, Y. S., Lee, C. K., & Han, S. S. (1994). Antimicrobial activity of *Ganoderma lucidum* extract alone and in combination with some antibiotics. *Archives of Pharmacal Research, 17*(6), 438–442.

Zhang, L., & Chen, J. (1997). Isolation and determination of molecular weights of water-soluble polysaccharides from fruiting body of *Ganoderma lucidum*. *Acta Polymerica Sinica, 1*(2), 68–72.

Zhang, J., Wang, G. Y., Li, H., Zhuang, C., Mizuno, T., Ito, H., et al. (1994). Antitumor active protein-containing glycans from the Chinese mushroom Songshan lingzhi, *Ganoderma tsugae* mycelium. *Bioscience Biotechnology and Biochemistry, 58*(7), 1202–1205.

Zhu, K. X., & Zhou, H. M. (2005). Purification and characterization of a novel glycoprotein from wheat germ water-soluble extracts. *Process Biochemistry, 40*(3–4), 1469–1474.