Carbohydrate Polymers 121 (2015) 183–189

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Structural characterization and antioxidant activity of a heteropolysaccharide from *Ganoderma capense*

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ARTICLE INFO

Article history:
Received 31 July 2014
Received in revised form 5 November 2014
Accepted 14 November 2014
Available online 24 November 2014

Keywords:
Ganoderma capense
Polysaccharide
Structural characterization
Antioxidant activity.

ABSTRACT

In this work, crude polysaccharide extracts were obtained from mycelia of the edible fungus *Ganoderma capense* (Lloyd) Teng. After removal of proteins by the Sevage method, fractionation and purification by anion-exchange and gel-permeation chromatography, a polysaccharide (GCPB-3) was isolated. The relative molecular weight of GCPB-3 was 124 kDa determined by high performance gel permeation chromatography (HPGPC). The homogeneous polysaccharide was composed of D-xylose and L-arabinose in the ratio of 1:1, and showed a specific optical rotation of $[\alpha]_D^{25} = +145^\circ$ (c 1.0, H_2O). Its structural features were determined by monosaccharide analysis, partial acid hydrolysis, methylation analysis, periodic acid oxidation, gas chromatography-mass spectrometry (GC-MS), Fourier transform-infrared spectroscopy (FT-IR) and nuclear magnetic resonance (NMR) spectroscopy (^1H, ^{13}C, HMQC and HMBC). The results characterized GCPB-3 as a heteropolysaccharide with backbone consisting of β-L-Ara*p* and β-D-Xyl*p*, linked with 1→4 sugar bonds. Interestingly, GCPB-3 showed some DPPH•- and hydroxy-radical scavenging activities.

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1. Introduction

Ganoderma is one of the most important edible fungi, and belongs to the class of basidiomycetes and the family *Polyporaceae* (Sun et al., 2006). Fungi of the genus *Ganoderma* have been used widely in many Asian countries such as China, Japan and Korea for thousands of years (Yang, Wang, Xie, Sun, & Wang, 2010). They are called 'Lingzhi' in Chinese and 'Reishi' in Japanese, and have been collected, cultivated and used as healthy functional food, herb of longevity, as well as traditional medicine. The most commonly used species include *G. capense*, *G. lucidum* and *G. Japonicum*. *Ganoderma* is famous and precious for containing a wealth of valuable polysaccharides, triterpenoids (Chen et al., 2012), proteins, amino acids, alkaloids, steroids, lactones, nucleotides, fatty acids, enzymes and lectins (Berovič et al., 2003; Han et al., 2012). Indeed, these chemical compounds have been isolated from the mycelia and fruiting bodies of *Ganoderma* mushrooms, and showed various pharmacological activities against aging, malignant tumors, hypertension, hypercholesterolemia, lupus erythematosus, hepatitis B, sclerema adultorum, alopecia reata, myodystrophy, insomnia and atopic dermatitis (Ngai & Ng, 2004; Li, Yan, Hua, & Zhang, 2013; Pan, Jiang, Liu, Miao & Zhong, 2013). As healthy functional food, *Ganoderma* tastes bitter and is appetizing with most nutrients easily obtained by ordinary cooking methods. *G. capense* is a *Ganoderma* species that morphologically resembles *G. lucidum* to a certain extent. In contrast to *G. lucidum*, fewer studies have focused on *G. Capense*, and mostly assessed glycopeptides. As a tradition Chinese medicine, *G. capense* is used to slow down the aging of organs and prolong life (Shi, Yang, Hu & Zhang, 2014). It was hypothesized that the antioxidant activity of *G. capense* should be one of the reasons for their anti-aging activity. In our previous studies, the four crude polysaccharides of *G. capense* had DPPH• radicals scavenging activities (Li, Yan, Hua, & Zhang, 2013; Yan, Kong, Zhang, & Cui, 2013), and the crude polysaccharide PB has noticeable effect at a high concentration, which was similar closed to the positive control (vitamin C). Therefore, it is essential and meaningful to investigate the exact structures of polysaccharides in PB and their antioxidant activities (Zhang, Liu, Park, Xia, & Kim, 2012).

Polysaccharides are polymers that consist of more than 10 monosaccharides linked by glycosidic bonds. They are widely found in plants, microorganisms, algae and animals, and show unique biological activities for cardiovascular diseases, hepatitis and cancer prevention and treatment, and in the regulation of glucose metabolism (Xu et al., 2009). With the continuous progress of modern science and technology, further analytical methods have been developed to improve the study of polysaccharides. In recent years, a deeper understanding of natural and effective methods has promoted studies dealing with the physical and chemical

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http://dx.doi.org/10.1016/j.carbpol.2014.11.034
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characterization of polysaccharides. To further research and development of homogeneous polysaccharides, a special focus on their structural analysis and biological activities is of great value.

In all living organisms, oxidation is an essential process that produces the energy necessary for biological processes (Duan, Zhang, Li, & Wang, 2006; Kong et al., 2010). However, uncontrolled and over-production of oxygen-derived free radicals cause many diseases such as degenerative processes, rheumatoid arthritis, atherosclerosis and cancer (Mau, Lin, & Song, 2002). Indeed, free radicals are able to damage numerous biological substances, including DNA, proteins and lipid membranes (Tsai, Song, Shih, & Yen, 2007). Meanwhile, it was reported that synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tert-butylated hydroxyquinone (TBHQ) promoted tumor formation (Cheung, Cheung, & Ooi, 2003). Interestingly, polysaccharides are somewhat resistant to oxidation and can be used as new antioxidants for their nontoxicity (Chen, Xie, Nie, Li, & Wang, 2008).

2. Materials and methods

2.1. Materials and chemicals

Submerged fermentation mycelium powder of *G. capense* was provided by Huai'an Yutu *Ganoderma* Co. Ltd. (Jiangsu Province, China). DEAE cellulose-52 was obtained from Whatman Ltd. Sephadex G-75 gel was purchased from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). T-series dextrans, trifluoroacetic acid (TFA), DPPH· (1,1-diphenyl-2-picrylhydrazyl) and standard monosaccharides were purchased from Sigma-Aldrich (St. Louis, USA). Phenol, glucose and sulfuric acid were supplied by Guangzhou Reagent Co. (Guangzhou, China). All other chemicals and reagents were of analytical grade.

2.2. Extraction and purification

G. capense mycelium powder (3.8 kg) was placed in round bottom flask and soaked with petroleum ether (five times v/w) overnight. The powder was dried and extracted three times with 38 L water at 70 °C for 3 h each time, thereafter, the extract was filtrated. Then, the residual *G. capense* mycelium powder was immersed in 0.3 M NaOH solution (w/v 1:15). A centrifugation at 5000 r/min for 5 min was carried out after stirring for 2 h at room temperature. The resulting supernatant was neutralized by 0.5 M HCl, followed by addition of ethanol to a final concentration of 70%. The pellet obtained after centrifugation was named PB. The polysaccharides samples (PB) were deproteinized by Sevage reagent (1-butanol/chloroform, v/v = 1:4). One liter water solution of polysaccharide and 200 mL Sevage reagent were mixed together in a separating funnel. After vibrating for 10 min and placing for 2 h, denatured protein was outflowed from the separating funnel. Repeat adding Sevage reagent and vibrating until little protein was left. The crude polysaccharide extracts were obtained after dialysis (M_W cut off: 3000 Da) and lyophilization.

The crude polysaccharide extracts were dissolved in distilled water, centrifuged and filtrated. The supernatant was then fractionated on a DEAE cellulose-52 column (ϕ2.6 × 40 cm), with 0.05, 0.1 and 0.25 M sodium chloride as elution gradient. An automated step-by-step fraction collector was used for collection of test tubes. All fractions were analyzed for carbohydrate content using the phenol-sulfuric method (Dubois, Gilles, Hamilto, Rebers, & Smith, 1956). Three peaks were obtained at 490 nm and the main fractions containing carbohydrates were collected, concentrated, dialyzed, lyophilized and denoted as PB-1, PB-2 and PB-3, respectively. PB-3 was then applied to a Sephadex G-75 gel-filtration column (ϕ1.6 × 100 cm), and eluted with deionized water at a flow rate of 0.3 mL/min for further purification. At this time, only one sharp peak was obtained by measuring absorbance at 490 nm, and this fraction was named GCPB-3.

2.3. Optical rotation identification

GCPB-3 was dissolved in deionized water completely. After addition of ethanol to a final concentration of 50% for 12 h in 25 °C and centrifugation at 3500 r/min for 5 min, a precipitate was obtained. Subsequently, using the same method, ethanol was added to the supernatant to a final concentration of 70%. These two precipitates were dissolved in distilled water to the same concentration after lyophilization. Optical rotation was determined with a P8000 Kruss polarimeter (Germany).

2.4. Homogeneity and molecular weight determination

High performance gel permeation chromatography (HPGPC) (Sun et al., 2010a) was used for the determination of homogeneity and molecular weight of GCPB-3. This was performed on a high performance liquid chromatography system equipped with TSK-GEL G-5000PW$_{XL}$ and G-3000PW$_{XL}$ gel columns in series (Tosoh Biosep, Japan), using 0.02 M monopotassium phosphate solution as eluent at a flow rate of 0.6 mL/min. Detection was by a Waters 2414 refractive index detector (Massachusetts, USA). The column was calibrated with the Dextran T-series standard of different molecular weights (Dextran T1000, T500, T70, T40, T10 and T5) and the column was kept at 35 °C during the experiment. The following standard curve equation was obtain by Breeze GCP Soft, Log $M_W = 8.17e+001 - 1.34e+001\,V + 7.87e-001\,V^2 - 1.58e-002\,V^3$, where V was elution volume (Geng, Chen, & Xu, 2009). Finally, the molecular weight of GCPB-3 was estimated using the calibration curve prepared above.

2.5. Analysis of monosaccharide composition

Identification and quantification of the monosaccharides of GCPB-3 were carried out by gas chromatography (GC). The sample (5 mg) was hydrolyzed with 2 mL TFA (2 M) at 120 °C for 6 h in a sealed glass tube. After evaporation to dryness, methanol was added to the sample, and the resulting solution was evaporated repeatedly to dryness till neutral pH was obtained. Afterward, hydroxylamine hydrochloride (10 mg) and 1 mL pyridine were added for 30 min at 90 °C for acetylization. Acetic anhydride (1 mL) was then added with continuous heating, and alditol acetate derivatives were obtained and analyzed on an Agilent 6820 GC system (Agilent, USA) equipped with an OV-17 capillary column. The temperature program of the column was set as follows: initial temperature of 200 °C was increased to 220 °C at a rate of 15 °C/min, and kept for 10 min, then, it was increased from 220 °C to 240 °C at 10 °C/min, and kept for 10 min at 240 °C. The injection temperature was 250 °C finally.

2.6. Fourier transform-infrared (FT-IR) spectrometry

GCPB-3 (2 mg) was incorporated, grounded and pressed into a pellet with potassium bromide. Spectra were recorded at absorbance mode from 4000 to 400 cm^{-1} with a Perkin-Elmer FT-IR spectrometer.

2.7. Periodate oxidation and Smith degradation

GCPB-3 (30 mg) was oxidized with 30 mL sodium periodate ($NaIO_4$, 0.015 M) in darkness at 4 °C. The reaction was completed when absorbance did not decrease as monitored at 223 nm every

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