Carbohydrate Polymers 80 (2010) 377–380

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

Structural characterization and hydroxyl radicals scavenging capacity of a polysaccharide from the fruiting bodies of *Auricularia polytricha*

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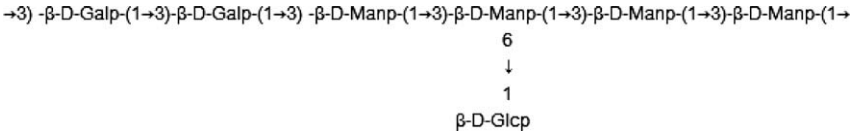
ARTICLE INFO

Article history:
Received 26 October 2009
Received in revised form 11 November 2009
Accepted 18 November 2009
Available online 24 November 2009

Keywords:
Auricularia polytricha
Polysaccharide
Structural characterization
Hydroxyl radicals scavenging

ABSTRACT

One low molecular-weight polysaccharide (APP) of 2.8×10^4 Da was isolated from the fruiting body of *Auricularia polytricha*. Partial hydrolysis with acid, periodate oxidation–Smith degradation, acetylation, methylation analysis and NMR spectroscopy (^{1}H, ^{13}C) were conducted to elucidate its structure and the repeating unit of APP was established as: the backbone was composed of (1 → 3)-linked-β-D-mannopyranosyl, (1 → 3)-linked-β-D-galactopyranosyl and (1 → 3,6)-linked-β-D-mannopyranosyl residues in the ratio of 3:2:1, and terminated with one single terminal (1→)-β-D-glucopyranosyl at the *O*-6 position of (1 → 3,6)-linked-β-D-mannopyranosyl along the main chain.



Preliminary antioxidant activity test in vitro showed APP could potentialize scavenging effect on hydroxyl radicals in a dose dependent manner.

1. Introduction

Recently, mushroom polysaccharides and their conjugates, used in health-care food or medicine, have drawn the attention of biochemical and nutritional researchers, due to their various biological activities (Sun & Liu, 2008). However, synthetic antioxidants used to reduce damage to the human body at the present time are suspected of being responsible for liver damage and carcinogenesis (Grice, 1988). Published data indicated that most polysaccharides derived from plants are relatively nontoxic and do not cause significant side effects. Thus, it is essential to develop effective natural antioxidants to protect the human body from oxidative damage.

Auricularia polytricha, which belongs to Auriculariaceae family, is widespread in many districts of China, such as Heilongjiang, Hebei, Jiangxi, Anhui and Guangdong province. The modern pharmacology research indicated that it has the functions of lowering blood-fat, antioxidant, antitumor, antinociceptive and immunomodulatory activities (Luo et al., 2009; Mau, Chao, & Wu, 2001; Yang et al., 2002). However, up to now, no detailed investigation has been conducted on structural characterization and antioxidative capacity of polysaccharides isolated from *A. polytricha*. Thus we initiated this research on the structural elucidation of a polysaccharide from the fruiting body of *A. polytricha* and explored its scavenging effect on hydroxyl radicals for seeking new biological functional principle used in food and pharmaceutical industry.

2. Materials and methods

2.1. Chemicals

DEAE–cellulose, Sephadex G-100 and Sephadex G-25 were purchased from Amersham (Sweden). T-series dextran, dimethyl sulfoxide (DMSO), standard sugars, deoxyribose, ferrous ammonium sulfate, ethylene diamine tetraacetic acid (EDTA), H_2O_2, ascorbate acid, and thiobarbituric acid (TBA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All of other reagents were analytical grade from Peking Chemical Co. (Peking, China).

2.2. General methods

UV–Vis absorption spectra were recorded with a UV–vis spectrophotometer (Model SP-752, China). GC was performed on a

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doi:10.1016/j.carbpol.2009.11.033

Shimadzu GC-14C instrument (Shimadzu, Japan) equipped with DB-1 capillary column (30 m × 0.25 mm × 0.25 μm). Gas chromatography–mass spectrometry (GC–MS) was done on a Shimadzu QP-2010 instrument (Shimadzu, Japan) with an HP-5MS quartz capillary column (30 m × 0.25 mm × 0.25 μm). The FTIR spectra (KBr pellets) were recorded on SPECORD in a range of 400–4000 cm^{-1}. The total carbohydrate content was determined by phenol–H_2SO_4 method using glucose as standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Protein was measured by the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951), using bovine serum albumin as standard. Dialysis was carried out using tubing with Mw cut-off 500 Da (for globular protein).

2.3. Extraction and purification of polysaccharide

The fruiting body of *A. polytricha* (0.5 kg) was extracted with distilled water (8000 mL × 3) at 90 °C for three times and 3 h for each time. After centrifugation (1700*g* for 10 min, at 20 °C), the supernatant was concentrated 10-fold, and precipitated with 5 volumes of dehydrated alcohol at 4 °C overnight. The precipitate collected by centrifugation was deproteinated by a combination of proteinase and Sevag method (Sun et al., 2008) and exhaustively dialyzed against water for 48 h. Then the concentrated dialyzate was precipitated with 5 volumes of dehydrated alcohol. The precipitate was washed with absolute ethanol, acetone and ether, respectively. The washed precipitate was the crude polysaccharide, named as CAPP (36.2 g).

The CAPP was purified on the auto liquid chromatographic fractionation apparatus (MF99-3) made in shanghai city of China. The CAPP was dissolved in distilled water, centrifuged, and then the supernatant was loaded onto a column of DEAE–cellulose anion-exchange chromatography column (3 × 40 cm). After loading with sample the column was eluted with distilled water and a gradient of NaCl aqueous solution (0 and 2 M) stepwise at 1 mL/min. Different fractions were collected using an automated fraction collector. The eluted solution by distilled water was only separated into one fraction, and was further purified on a Sepharose G-100 column (2.6 × 100 cm) with 0.15 mol/L NaCl at a flow rate of 0.5 mL/min to yield only one fraction, termed as APP. Total carbohydrate and protein content of each tube was measured by Dubois's and Lowry's method, respectively.

2.4. Determination of monosaccharide composition and molecular weight

Gas chromatography (GC) was used for identification and quantification of the monosaccharides. APP was hydrolyzed with 2 M TFA at 120 °C for 2 h. The monosaccharides were conventionally converted into the alditol acetates as described (Johnes & Albersheim, 1972; Oades, 1967) and analyzed by GC as foresaid. The absolute configurations of the monosaccharides were determined as described by Vliegenthart and co-workers using (+)-2-butanol (Gerwig, Kamerling, & Vliegenthart, 1979).

The average molecular weight of APP was determined by high-performance size-exclusion chromatography (HPSEC) (Sun et al., 2008), which was performed on a SHIMADZU HPLC system fitted with one TSK-G3000PW_{XL} columns (7.8 mmID × 30.0 cm) and a SHIMADZU RID-10A detector. The data were processed by GPC processing software (Millennium^{32} version). The mobile phase was 0.7% Na_2SO_4, and the flow rate was 0.5 mL min^{-1} at 40 °C, with 1.6 mpa. A sample (3 mg) was dissolved in the mobile phase (0.5 mL) and centrifuged (10,000 rpm, 3 min), and 20 μL of supernatant was injected in each run. The molecular mass was estimated by reference to a calibration curve made from a set of Dextran T-series standards of known molecular mass (T-130 80, 50, 25, 10).

2.5. Partial acid hydrolysis

The APP (100 mg) was hydrolyzed with 0.05 M trifluoroacetic acid (3 mL) at 95 °C for 10 h, and then centrifuged, After TFA was removed by evaporation, the remains was dialyzed with distilled water for 48 h, and then diluted the solution in the sack with ethanol. After hydrolyzation, the precipitate and supernatant in the sack and the fraction out of sack were dried, analyzed by GC as the alditol acetate. The precipitate, in the sack, was subjected to monosaccharide composition analysis of backbone and methylation analysis (Sun & Liu, 2008).

2.6. Periodate oxidation and Smith degradation

For analytical purpose, 25 mg of the polysaccharide were dissolved in 12.5 mL of distilled water and 12.5 mL of 30 mmol/l $NaIO_4$ were added. The solution was kept at 4 °C for 7 days in the dark, 0.1 mL aliquots were withdrawn at 3–6 h intervals, diluted to 25 mL with distilled water and read in a spectrophometer at 223 nm (Linker, Evans, & Impallomeni, 2001). Excess periodate was decomposed by addition of ethylene glycol (2 mL), The solution of periodate product (2 mL) was sampled to calculate the yield of formic acid by 0.01 M NaOH, The rest was dialyzed against distilled H_2O for 24 h. The solution was concentrated and reduced with $NaBH_4$ (60 mg), and the mixture was left for 24 h at room temperature, neutralized to pH 6.0 with 50% acetic acid, dialyzed as described above, and concentrated to a volume (10 mL). One-third of solution described above was freeze-dried and analyzed with GC. Others were added to the same volume of 1 M sulfuric acid, kept for 40 h at 25 °C, neutralized to pH 6.0 with barium carbonate, and filtered. The filtrate was dialyzed as foresaid, and the content out of sack was desiccated for GC analysis; the content inside was diluted with ethanol, and after centrifugation, the supernatant and precipitate were also dried out for the GC analysis.

2.7. Methylation analysis

The sample (20 mg) was methylated thrice according to the Needs and Selvendran (1993). Complete methylation was confirmed by the disappearance of the OH band (3200–3700 cm^{-1}) in the IR spectrum. The methylated products were hydrolyzed, reduced and acetylated as described by Sweet, Shapiro, and Albersheim (1975). The partially methylated alditol acetates were analyzed by GC–MS under the same chromatographic conditions, as described above.

2.8. NMR spectroscopy

For NMR measurements APP was dried in a vacuum over P_2O_5 for several days, and then exchanged with deuterium by lyophilizing with D_2O for several times (Dueñas-Chasco et al., 1997). The deuterium-exchanged polysaccharide (50 mg) was put in a 5-mm NMR tube and dissolved in 0.7 mL 99.96% D_2O. Spectra were recorded with a Bruker AV-400 spectrometer. The ^1H and ^{13}C NMR spectra were recorded at 50 °C. Acetone was used as an internal standard (δ31.07 ppm) for the ^{13}C spectrum. The ^1H NMR spectrum was recorded fixing the HOD singal at δ4.58 ppm at 50 °C.

2.9. Assay for the scavenging effect on hydroxyl radicals

In vitro antioxidant activity was evaluated using the hydroxyl radical system generated by the Fenton reaction (Heo, Park, Lee, & Jeon, 2005) with a minor modification. Briefly, Samples were dissolved in distilled water at 0 (control), 0.5, 1, 2, 4, and 8 mg/mL. The reaction mixture contained 1 mL of brilliant green (0.435 mM), 0.5 mL of $FeSO_4$ (2 mM), 1.5 mL of H_2O_2 (3.0%) and 1 mL samples of varying concentrations. After incubation at room

Table 1
The data of UV analysis, IR analysis and NMR analysis of APP.

Assay	Peaks or signals at
UV analysis	210 nm, 260 nm, 280 nm
IR analysis	3411.05 cm^{-1}; 2923.24 cm^{-1}; 1623.33 cm^{-1}; 890.21 cm^{-1}
^{1}H NMR analysis	4.82 ppm; 4.80 ppm; 4.78 ppm; 4.66 ppm
^{13}C NMR analysis	105.75 ppm; 104.58 ppm; 100.60 ppm; 100.41 ppm; 82.32 ppm; 78.65 ppm; 77.56 ppm; 72.34 ppm; 68.77 ppm; 62.58 ppm; 62.14 ppm; 60.15 ppm;

temperature for 20 min, the absorbance of the mixture was measured at 624 nm. Hydroxyl radicals gave a brilliant green colour, so the absorbance change of the reaction mixture indicated the scavenging ability for hydroxyl radicals. The hydroxyl radical-scavenging rate was calculated as (1–absorbance of sample/absorbance of control) × 100%.

3. Results and discussion

3.1. Isolation, purification and structural analysis of polysaccharides

The APP showed a single and symmetrically sharp peak, indicating its homogeneity on HPSEC (data not shown), According to the retention time, its molecular weight was estimated to be 2.8×10^4 Da. It had a negative response to the Bradford test and no absorption at 280 or 260 nm in the UV spectrum, indicating the absence of protein and nucleic acid. Total carbohydrate content was determined to be 91%. The APP was composed of D-mannose, D-galactose and D-glucose as detected by GC with molar ratios of 4:2:1.

The FTIR spectra of APP are shown in Table 1. The bands in the region of 3411.05 cm^{-1} were due to the hydroxyl stretching vibration of the polysaccharides. The bands in the region of 2923.24 cm^{-1} were due to C–H stretching vibration, and the bands in the region of 1623.33 cm^{-1} were due to associated water. Moreover, the characteristic absorptions at 890.21 cm^{-1} indicated β-configurations existing in APP.

The GC–MS results (Table 2) indicated that the backbone chain was mainly (1 → 3)-linked-β-D-mannopyranosyl (Residue-A), (1 → 3)-linked-β-D-galactopyranosyl residues (Residue-B) and (1 → 3, 6)-linked-β-D-mannopyranosyl (Residue-C). The side chain attached to the *O*-6 position of Residue-C contained single terminal (1 →)-β-D-glucopyranosyl (Residue-D) group. According to the peak areas, four types of residues were in the ratio of 3:2:1:1. This was also in accordance with the results of the periodate oxidation and Smith degradation. Supporting the results of methylation analysis, GC of the products that was obtained from periodate oxidation and Smith degradation showed the presence of glycerol, mannose and galactose. GC quantification showed that both the original mannose and galactose totally had survived oxidation (data not shown).

In the anomeric region of the ^{1}H NMR spectrum (Table 1) of APP, three signals occurred at δ4.78, δ4.82, δ4.80 and δ4.66 ppm, which were assigned as Residue-A, Residue-B, Residue-C and Residue-D, respectively. And accordingly in the anomeric region of the ^{13}C NMR spectrum (Table 1), four carbon resonances appeared at δ100.41, δ104.58, δ100.60 and δ105.75 ppm. All the results confirmed the presence of four sugar residues and their configurations: Residue-A, B, C and D are β-configurations, consistent with GC and FTIR data. In the high magnetic field, the δ78.65, δ82.32, δ77.56 and δ72.34 signal should come from C-3 resonance of Residue-A, B, C and D. C-6 chemical shifts of Residue-A, Residue-B, Residue-C and Residue-D occurred at δ60.15, δ62.14, δ68.77 and δ62.58, respectively. All the NMR chemical shifts were compared with the literature values (Ali, Weintraub, & Widmalm, 2006; Mondal, Chakraborty, Rout, & Islam, 2006; Sun, Liang, Zhang, Tong, & Liu, 2009; Sun et al., 2008).

Table 2
The results of methylation analysis of APP.

Peak No.	Methylated sugar	Molar ratio	Linkage type
1 (Residue-A)	2,4,6-Me_3-Man*p*	3	→3)-β-Man*p*-(1→
2 (Residue-B)	2,4,6-Me_3-Gal*p*	2	→3)-β-Gal*p*-(1→
3 (Residue-C)	2,4-Me_3-Man*p*	1	→3, 6)-β-Man*p*-(1→
4 (Residue-D)	2,3,4,6-Me_4-Glc*p*	1	β-Glc*p*-(1→

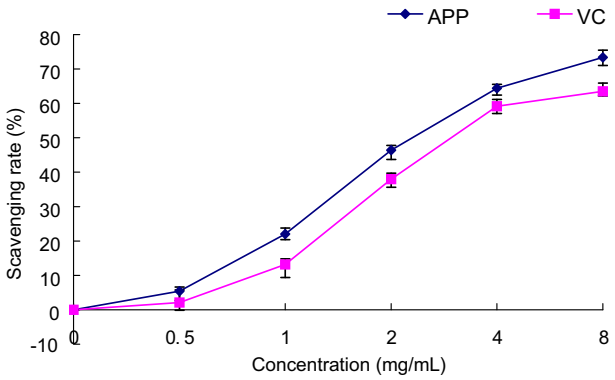

Fig. 1. Hydroxyl radical-scavenging activity of APP and Vc. Values are means ± SD of three determinations.

The structure characterization of a polysaccharide from the fruiting body of *A. polytricha*, by means of a combination of chemical and instrumental analysis, was studied for the first time. The structure of this polysaccharide can be demonstrated as follows: the backbone of APP was composed of (1 → 3)-linked-β-D-mannopyranosyl, (1 → 3)-linked-β-D-galactopyranosyl and (1 → 3, 6)-linked-β-D-mannopyranosyl residues in the ratio of 3:2:1, and terminated with one single terminal (1 →)-β-D-glucopyranosyl at the *O*-6 position of (1 → 3, 6)-linked-β-D-mannopyranosyl along the main chain.

3.2. Scavenging activity of APP on hydroxyl radical

Generation of Reactive oxygen species (ROS) beyond the body's antioxidant capacity gives rise to oxidative stress. Much of the oxidative damage to biomolecules can be induced by ·OH, the most reactive one among ROS species (Yang, Liu, Han, & Sun, 2006). As illustrated in Fig. 1, the hydroxy radical-scavenging activity initiated by APP is stronger than Vc at the same conditions (from 0.5 to 8 mg/mL.) in a concentration-dependent manner. This result proved that APP had significant effect on scavenging hydroxyl radical. Because of the complex mechanism of antioxidant activity, one test is normally not enough to evaluate precisely the antioxidant activity of the potential antioxidant. So various oxidative stress-mediated injury models for APP should deserve more an in-depth research in the future.

Acknowledgements

This work was financially supported by the Natural Science Foundation for Young Scientists of Heilongjiang Province, China (Grant No. QC2009C106).

References

Ali, T., Weintraub, A., & Widmalm, G. (2006). Structural determination of the *O*-antigenic polysaccharide from the Shiga toxin-producing *Escherichia coli* O171. *Carbohydrate Research, 341*, 1878–1883.

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

Dueñas-Chasco, M. T., Rodríguez-Carvajal, M. A., Mateo, P. T., Franco-Rodríguez, G., Espartero, J., Irastorza-Iribas, A., et al. (1997). Structural analysis of the exopolysaccharide produced by *Pediococcus damnosus*. *Carbohydrate Research, 303*, 453–458.

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.

Grice, H. C. (1988). Safety evaluation of butylated hydroxyanisole from the perspective of effects on forestomach and oesophageal squamous epithelium. *Food and Chemical Toxicology, 26*, 717–723.

Heo, S. J., Park, E. J., Lee, K. W., & Jeon, Y. J. (2005). Antioxidant activities of enzymatic extracts from brown seaweeds. *Bioresource Technology, 96*, 1613–1623.

Linker, A., Evans, L. R., & Impallomeni, G. (2001). The structure of a polysaccharide from infectious strains of *Burkholderia cepacia*. *Carbohydrate Research, 335*, 45–54.

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

Luo, X., Yu, M. Y., Jiang, N., Xu, X. Y., Zeng, J., & Zheng, L. Y. (2009). Effects of *Auricularia polytricha* polysaccharide on mouse macrophage cytokine and iNOS gene expression. *Jun Wu Xi Tong, 28*, 435–439.

Johnes, T. M., & Albersheim, P. (1972). A gas chromatographic method for the determination of aldose and uronic acid constituents of plant cell wall polysaccharide. *Plant Physiology, 49*, 926–936.

Mau, J. L., Chao, G. R., & Wu, K. T. (2001). Antioxidant properties of methanolic extracts from several ear mushrooms. *Journal of Agricultural and Food Chemistry, 49*, 5461–5467.

Mondal, S., Chakraborty, I., Rout, D., & Islam, S. S. (2006). Isolation and structural elucidation of a water-soluble polysaccharide (PS-I) of a wild edible mushroom, *Termitomyces striatus*. *Carbohydrate Research, 341*, 878–886.

Needs, P. W., & Selvendran, R. R. (1993). Avoiding oxidative degradation during sodium hydroxide/methyl iodide-mediated carbohydrate methylation in dimethyl sulfoxide. *Carbohydrate Research, 245*, 1–10.

Oades, J. M. (1967). Gas-liquid chromatography of alditol acetates and its application to the analysis of sugars in complex hydrolysates. *Journal of Chromatography, 28*, 246–252.

Sun, Y. X., Liang, H. T., Zhang, X. T., Tong, H. B., & Liu, J. C. (2009). Structural elucidation and immunological activity of a polysaccharide from the fruiting body of *Armillaria mellea*. *Bioresource Technology, 100*, 1860–1863.

Sun, Y. X., & Liu, J. C. (2008). Structural characterization of a water-soluble polysaccharide from the Roots of *Codonopsis pilosula* and its immunity activity. *International Journal of Biological Macromolecules, 43*, 279–282.

Sun, Y. X., Wang, S. S., Li, T. B., Li, X., Jiao, L. L., & Zhang, L. P. (2008). Purification, structure and immunobiological activity of a new water-soluble polysaccharide from the mycelium of *Polyporus albicans*(Imaz) Teng. *Bioresource Technology, 99*, 900–904.

Sweet, D. P., Shapiro, R. H., & Albersheim, P. (1975). Quantitative analysis by various g.l.c. Response-factor theories for partially methylated and partially ethylated alditol acetates. *Carbohydrate Research, 40*, 217–225.

Yang, B. K., Ha, J. Y., Jeong, S. C., Jeon, Y. J., Ra, K. S., Das, S., et al. (2002). Hypolipidemic effect of an exo-biopolymer produced from submerged mycelial culture of *Auricularia polytricha* in rats. *Biotechnology Letters, 24*, 1319–1325.

Yang, Y., Liu, W. S., Han, B. Q., & Sun, H. Z. (2006). Antioxidative properties of a newly synthesized 2-glucosamine-thiazolidine-4(R)-carboxylic acid ($GlcNH_2Cys$) in mice. *Nutrition Research, 26*, 369–377.