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Structural analysis of anti-tumor heteropolysaccharide GFPS1b from the cultured mycelia of *Grifola frondosa* GF9801

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Abstract

A 21-kDa heteropolysaccharide, coded as GFPS1b, was obtained from the cultured mycelia of *Grifola frondosa* GF9801 by hot-water extraction, ethanol precipitation, and fractioned by DEAE Sepharose Fast-flow, followed by the purification with Sephadex G-100 column chromatography using an AKTA purifier. It exhibited more potent anti-proliferative activity on MCF-7 cells than other polysaccharide fractions. GFPS1b was an acidic polysaccharide with approximately 16.60% protein and 4.3% uronic acid. Gas chromatography of absolute acid hydrolysate of GFPS1b suggested that it was composed of D-glucose, D-galactose, and L-arabinose with a molar ratio of 4:2:1. Periodate oxidation, Smith degradation, partial acid hydrolyzation, methylation analysis, FT-IR, and ^{1}H, ^{13}C NMR spectroscopy analysis revealed that GFPS1b had a backbone consisting of α-(1 → 4)-linked D-galacopyranosyl and α-(1 → 3)-linked D-glucopyranosyl residues substituted at O-6 with glycosyl residues composed of α-L-arabinose–(1 → 4)-α-D-glucose (1→ linked residues.

Keywords: *Grifola frondosa*; Heteropolysaccharide; Structure analysis; Anti-tumor activity

1. Introduction

Medicinal mushrooms have been utilized in China, Japan, and other Asian countries for two thousand years as an edible and medical resource. Recently, a number of bioactive molecules, including anti-tumor substance, have been identified in numerous mushroom species (Mizuno et al., 1995). Polysaccharides, proteo-polysaccharides and their derivates from the cultured mushroom have been recognized to be the potent immuno-stimulatory and anti-tumor active compounds (Kim et al., 2003). Therefore, discovery and evaluation of new polysaccharides from the various medicinal mushrooms as new safe compounds for functional foods has become a hot research spot.

Grifola frondosa, an oriental fungus, has been reported to possess many biologically active compounds (Kawagishi et al., 1990; Lee et al., 2003; Ohno et al., 1985; Suzuki et al., 1984; Talpur et al., 2002). Especially, the anti-tumor and immuno-stimulating activities of its polysaccharide D-fraction, a branched β-(1 → 6)-D-glucan isolated from the fruiting body have been extensively studied (Kodama et al., 2002; Konno, 2004; Nanba, 1995). However, little attempt has been made to investigate the polysaccharides isolated from the cultured mycelia and their medicinal properties (Zhuang et al., 1994). We successfully isolated a novel heteropolysaccharide GFPS1b from the cultured mycelia of *G. frondosa*. To our knowledge, the characteristic structure of the GFPS1b has never reported yet, while the GFPS1b potentially has the selectively direct effect on the tumor cells MCF-7 in a dose-dependent manner. In the this work, we attempted to systematically investigate the separation, bioactivity-directed fractionation, purification and structural analysis of the anti-tumor active polysaccharide fraction GFPS1b from *G. frondosa* GF9801.

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2. Methods

2.1. Microorganism and growth medium

G. frondosa GF9801 was maintained on potato dextrose agar (PDA) slants and sub-cultured every two months. The mycelia were grown at 25 °C with shaking in Erlenmeyer flasks in the medium containing (g/l): glucose 45.2, KH_2PO_4 2.97, peptone 6.58, $MgSO_4 \cdot 7H_2O$ 1 and corn steep liquor 15 (Cui et al., in press). Mycelia were filtered, washed with distilled water, and maintained in a frozen condition.

2.2. Isolation and purification of polysaccharide GFPS1b

The mycelia of *G. frondosa* GF9801 were extracted with 1 l of water at 90 °C for 2 h and retreated two more times. The extracts were centrifuged at 5000*g* for 20 min. The supernatant obtained was concentrated under reduced pressure and then added 3 vols. EtOH with vigorous stirring. The solution was then maintained overnight at 4 °C. The precipitate was collected by centrifugation at 10,000*g* for 10 min and dissolved with H_2O. The soluble part was separated by a DEAE-Sepharose fast-flow anionic resin (Pharmacia AP, Sweden) column (25 × 5 cm, i.d.). Stepwise elution was performed with a discontinuous gradient of water, 0.1, 0.3 and 0.5 M NaCl at pH 7.0 (every fraction tube 6 ml). The carbohydrate content of the eluates was determined spectrophotometrically at 490 nm using the phenol–sulfuric acid method (Dubois et al., 1956).

The protein concentration of the column fractions was determined by measuring the absorption at 280 nm. Four factions, GFPS0, GFPS1, GFPS3 and GFPS5 were collected, dialyzed and lyophilized. The main anti-tumor activity was detected in the fraction GFPS1. Further purification of GFPS1 was implemented with gel filtration by a Sephadex G-100 (Amersham Biosciences, Sweden), using an AKTA purifier (Amersham Biosciences, Sweden). Two fractions of GFPS1a and GFPS1b were separated and then they were lyophilized for anti-tumor testing and the subsequent structural analysis.

2.3. Homogeneity and M_rs

The average molecular weight of GFPS1b was determined by HPGPC. The HPGPC system comprised a Rheodyne model 7725 sample injector, a 510 pump, a 2410 refractive index detector, a 740 data module, and a guard column (300 mm × 7.8 mm, Ultrahydrogel™ 500) (Waters, America) connected in series. The mobile phase was sodium acetate buffer (0.05 M, pH 3.65) with a flow rate 0.9 ml/min. The standards used to calibrate the column system included T-series Dextran (T-2000, T-580, T-190, T-70, and T-10, Sigma). Data analysis was performed using Millennium 2010 Software (Waters, America).

2.4. Quantitative determination of protein content and analysis of hydrolyzed amino acid

The protein content of the samples was determined by Micro BCA™ Protein Assay Reagent Kit (Shenergy Biocolor BioScience & Technology Co., Shanghai, China) using bovine serum albumin as the standard. For determination of amino acid composition of GFPS1b, it was subjected to hydrolysis under vacuum with 6 M HCl solution at 110 °C for 24 h. The hydrolysate was evaporated and then the dried residue was redissolved in 0.02 M HCl solution. The amino acid composition was determined using a Hitachi 835-50G automatic amino acid analyzer (Hitachi Ltd., Tokyo, Japan).

2.5. Monosaccharide analysis

Monosaccharide components and their ratios were determined by absolute hydrolysis. With this method, the sample was hydrolyzed with 1 M H_2SO_4 at 90 °C for 8 h and the hydrolysate was then neutralized with $CaCO_3$. The resulting solution was centrifuged, evaporated for dryness, and then followed by the acetylation treatment with Ac_2O-Pyridine at 90 °C for 30 min. The resulting alditol acetate was analyzed by a gas chromatography (model 3300, Varian Co. Ltd., USA) equipped with an OV 1701 capillary column (30 m × 0.32 mm i.d.), with helium as the carrier gas. The analysis was firstly carried out at the range of 150–240 °C with a temperature rising rate of 40 °C/min, and then the temperature was kept constant until the end of the analysis for 20 min. The products were identified by their characteristic retention times. Uronic acid contents were determined by measuring the absorbance at 525 nm using the *m*-hydroxybiphenyl colorimetric procedure and with D-glucuronic acid as the standard (Blumenkrantz and Asboe-Hansen, 1973).

2.6. Partial hydrolysis with TFA

Polysaccharide sample was hydrolyzed with 0.5 M trifluoroacetic acid (TFA) at 90 °C for 2 h, and then the hydrolysate was dialyzed with distilled water for 24 h. The solution in the sack was precipitated with ethanol. The monosaccharide analysis of the precipitate fractions, supernatant in the sack and the fraction out of sack was followed the same procedure as mentioned above.

2.7. Periodate oxidation and smith degradation

GFPS1b was added to 15 ml 0.015 M $NaIO_4$ solution in a round-bottom flask, and the mixture was kept at 4 °C in dark condition. Absorption at 223 nm was detected every 8 h. After the oxidation was completed (64 h), the excess periodate was reduced by adding ethylene glycol (1.0 ml), and the solution was dialyzed with distilled water for 1 h. The product was further reduced with $NaBH_4$ (30 mg), and then followed by acidification with acetic acid. The