Available online at www.sciencedirect.com
ScienceDirect
Bioresource Technology 98 (2007) 395–401
BIORESOURCE TECHNOLOGY

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

F.J. Cui [a,b], W.Y. Tao [a,b,*], Z.H. Xu [b], W.J. Guo [b], H.Y. Xu [b], Z.H. Ao [b], J. Jin [b], Y.Q. Wei [a]

[a] *The Key Laboratory of Industrial Biotechnology, Ministry of Education, Southern Yangtze University, Wuxi 214036, PR China*
[b] *Laboratory of Biopharmaceutics, School of Biotechnology, Southern Yangtze University, Wuxi 214036, PR China*

Received 22 August 2005; received in revised form 6 December 2005; accepted 14 December 2005
Available online 3 February 2006

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.

* Corresponding author. Address: The Key Laboratory of Industrial Biotechnology, Ministry of Education, Southern Yangtze University, Wuxi 214036, PR China. Tel.: +86 510 5862938; fax: +86 510 5869645.
E-mail addresses: fengjiecui@sytu.edu.cn (F.J. Cui), wytao@sytu.edu.cn (W.Y. Tao).

0960-8524/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2005.12.015

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

final periodate-reduced product was hydrolyzed by 2 M H_2SO_4 solution at 90 °C for 8 h, and then the alditol acetates were prepared and analyzed with the same analysis method mentioned before.

2.8. Methylation analysis

GFPS1b was methylated by the method of Hakomori (1964). The methylated polysaccharide was examined by IR spectroscopy. Non-appearance of the hydroxyl absorption peak identified the complete methylation. The fully methylated sample was converted into alditol acetates by hydrolysis with H_2SO_4 solution, reduction with $NaBH_4$, and acetylation with acetyl anhydride. The resulting product was subjected to linkage analysis by GC–MS. A Finnigan Trace (Finnigan Co., America) GC with OV 1701 column interfaced to a Finnegan mass selective detector was used for mass spectral identification of the GC components. Linkages were identified on the basis of relative retention time and fragmentation pattern.

2.9. FT-IR and ^1H, ^{13}C NMR

FT-IR (Necolit 5D × B) was used for detecting the sample functional groups. Sample GFPS1b was dissolved in D_2O at the concentration of 30 mg/ml for ^1H and ^{13}C NMR analysis. ^1H and ^{13}C NMR spectroscopy were performed with a Varian Inova 600 (Varian, America) at room temperature. The chemical shifts were expressed in ppm relative to the response of the internal standard, 3-trimethylsily-1-propaneulfoic acid (sodium salt).

2.10. Assay of the effect on tumor cell lines

The anti-proliferative activity of the polysaccharide fractions of *G. frondosa* GF9801 was determined as follows. The cell line MCF-7 was maintained in RPMI-1640 (Gibco, America) supplemented with 10% fetal bovine serum (FBS), 4 μg/ml insulin, 100 mg/l streptomycin, and 100 IU/ml penicillin at 37 °C in a humidified atmosphere of 5% CO_2. Cells (8×10^3/well) in their exponential growth phase were seeded into each well of a 96-well flat-bottomed culture plate and incubated for 24 h before addition of the samples. Incubation was carried out for another 48 h. The viability of cells was assessed in the MTT assay as described elsewhere. Briefly, the medium was aspirated and MTT was added to cells at a concentration of 0.5 g/l. Cells were incubated at 37 °C for 4 h and the formazan product was solubilized with dimethylsulfoxide (DMSO). The absorbance was detected in the microplate reader (Multiskan MK3, Labsystems, Finland) at 570 nm, and measured values were expressed as mean ± SD.

3. Results and discussion

In this study, the anti-tumor polysaccharides from *G. frondosa* GF9801 by bioactivity-directed fractionation was isolated. The potential anti-tumor activity was evaluated by the inhibitory ratio of proliferation of MCF-7 cells. The result showed that separation of the water extract from *G. frondosa* GF9801 mycelia with ion exchange chromatography on DEAE-Sepharose fast-flow yielded a neutral (un-adsorbed) fraction (GFPS0) and three other adsorbed fractions (GFPS1, GFPS3 and GFPS5) (Fig. 1). GFPS1 fraction of *G. frondosa* GF9801 demonstrated higher activity in the inhibitory effect on MCF-7 cells growth than those of the others in a dose dependent manner in vitro (Fig. 2). Further purification was performed by applying GFPS1 to gel filtration on Sephadex G-100 to obtain two sub-fractions, GFPS1a and GFPS1b (Fig. 3). The latter sub-fraction showed higher anti-proliferative activity (Fig. 4). Hence, the structure of the GFPS1b sub-fraction was further studied. GFPS1b could be eluted as a single peak and its apparent molecular weight was estimated as 21 kDa by HPGPC on Ultrahydrogel™ 500 column. This result also revealed that GFPS1b might be a homogeneous polysaccharide on the basis of its molecular weight and polarity. A BCA assay of GFPS1b indicated that the polysaccharide contained approximately 16.60% protein portion. Table 1 showed the composition and relative percentage of hydrolyzed amino acids, and the concentrations of arginine, aspartic acid and glutamatic acid were relatively higher with their relative percentage of 3.32%, 3.01% and 3.01%, respectively. GFPS1b contained about 81.32% carbohydrate determined by the phenol–sulfuric acid method and 4.25% uronic acid residue. Monosaccharide analysis showed that the molar ratio of glucose, galactose, and arabinose was approximately at 4:2:1. The GC analysis results of partial acid hydrolysis indicated that the main backbone of GFPS1b was composed by glucose and galactose, since the two monosaccharides were detected in the precipitation in the sack. The assay showed that the molar ratio of arabinose increased sharply in the fraction out of the sack, suggesting that arabinose could be the terminal residues.

GFPS1b consumed large amount of 0.015 mol/l sodium periodate during the oxidation at 4 °C in 48 h and formic acid liberated was also detected, indicating the existing of the 1→ linked or (1 → 6)-linked monosaccharides. The oxidized GFPS1b was reduced with sodium borohydride, and then completely hydrolyzed with acid. GC analysis of the hydrolyzed products detected a little glycerol and large amount of erythritol, implying some of the linkages 1→, (1 → 2)-, (1, →6)-, (1 → 2, 6)-, (1 → 4)- and (1 → 4, 6)-existed in the structure of GFPS1b. Furthermore, the detected glucose indicated that part of that was (1 → 3)–(1 → 2, 3)- and (1 → 3, 6)-linked which could not be oxidized.

The IR spectra for the sample GFPS1b was presented in Fig. 3. The bands in the region of 3393, 2930, and 1642 cm^{-1} were considered as the results of the hydroxyl stretching vibration of the polysaccharide, C–H stretching vibration, and the combined water, respectively. Absorption at 924 cm^{-1} was typical for D-glucose in pyranose

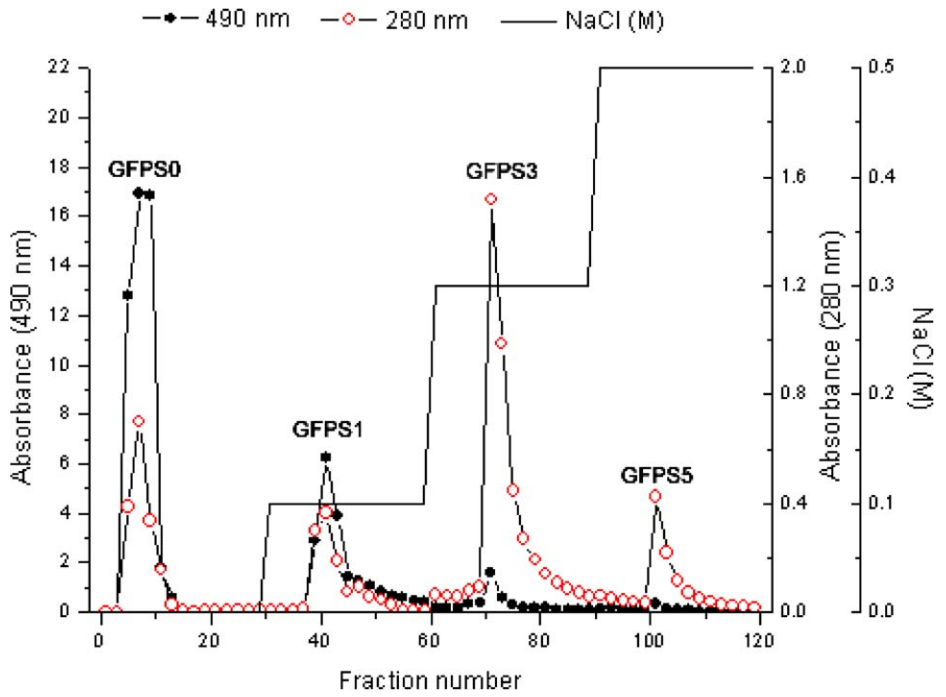

Fig. 1. Anion-exchange chromatography of polysaccharides from *G. frondosa* GF9801 on DEAE-Sepharose fast-flow. The column was eluted stepwise with H_2O, 0.1, 0.3 and 0.5 M NaCl solutions.

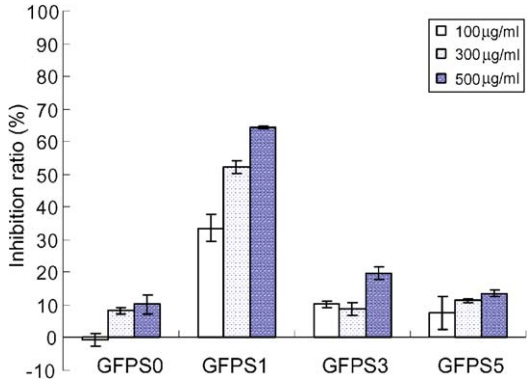

Fig. 2. Inhibition of proliferation of MCF-7 cells by different concentrations of polysaccharides from *G. frondosa* GF9801.

form, and 863 cm^{-1} was indicative index of α-glycosidic linkage between individual glycosyl residues existed in GFPS1b. The band of 1705 cm^{-1} was the characteristic carboxylic bond, which was in accordance with the results of colorimetric assay. The absorptions at 1075, 1219, and 1269 cm^{-1} also indicated a pyranose form of the glycosyl residue.

Per-methylated GFPS1b was obtained by the procedure of Hakomori. The per-methylated products were then hydrolyzed with acid, and the derivatized alditol acetates of the hydrolysate were analyzed with GC and GC–MS (Table 2). The molar ratios of 2,3,5-Me3-Ara, 2,3,6-Me3-Glu, 2,4,6-Me3-Glu, 2,3,6-Me3-Gal, 2,3,4-Me3-Gal and 2,4-Me2-Glu were 1.6, 1.2, 2.5, 1.0, 0.5 and 1.2 according to the peak areas. The ^{13}C NMR spectrum of GFPS1b and its chemical shift data (Table 3) showed that it contained carbohydrate and protein, with three major and two minor C-1 signals, a CO2–6 signal at 175.2 ppm. Based on the available data in the literatures (Mondal et al., 2004, 2005; Wang et al., 2005), the resonances in the region of 96–104 ppm were attributed to the anomeric carbon atoms of arabinofuranose (Ara*f*), galactopyranose (Gla*p*) and glucopyranose (Glc*p*), respectively. The resonance due to C-1 of Glc residues substituted at O-3 was observed at 101.7 ppm. The signals at 100.00 ppm correspond to C-1 Glc*p* residues linked by substitution at O-4. The peak at 104.1 ppm corresponded to C-1 of α-L-arabinofuranose residues. Others signals were assigned in Table 3. In the low field region (178 ppm), minor typical signal was observed for the carboxyl group of the uronic acid units, which was in agreement with the result from *m*-hydroxydiphenyl colorimetric method. In the proton spectrum, the three signals in the region of 4.99–5.25 ppm were assigned to anomeric protons of α-L-Ara*f*, α-D-Glc*p* and α-D-Gal*p* residues. The anomeric region of the ^1H spectrum (5.25–3.60 ppm) was consistent with the GFPS1b, which had a hexose-repeating unit.

Based on above results, it could be concluded that the backbone of GFPS1b contained →4)-Gal-(1→ and →3)-Glc-(1→. Residue of branched structure was (1 → 4)-linked glucose and the terminal residue of the structure was (1→ linked arabinose. It could also be speculated that GFPS1b was composed of a repeating unit probably having the structure as shown in Fig. 5, which was different from the reported structure of polysaccharide D-fraction isolated from fruiting bodies of *G. frondosa*. Here, the

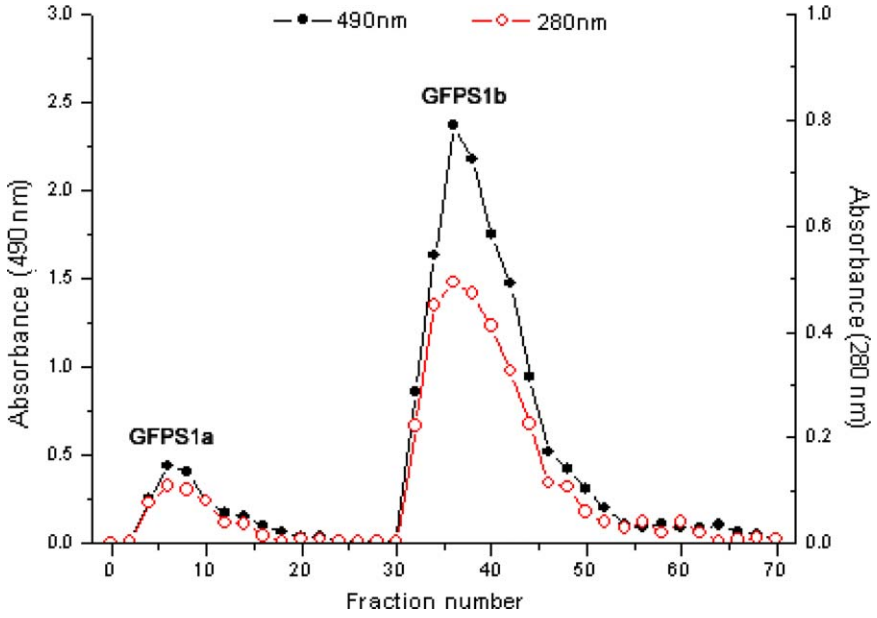

Fig. 3. Further purification of GFPS1 by gel filtration on a Sephadex G-100 using an AKTA purifier.

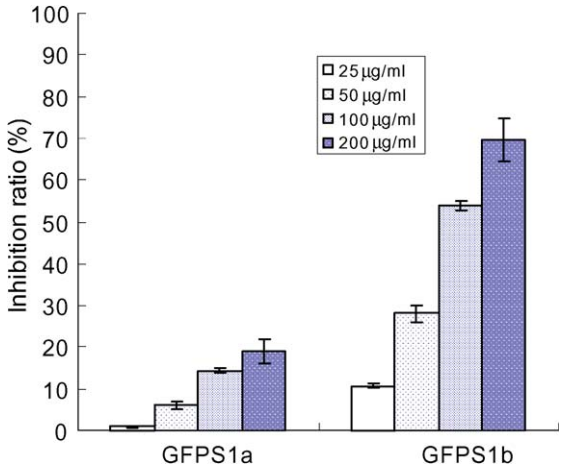

Fig. 4. Inhibition of proliferation of MCF-7 cells by different concentrations of GFPS1a and GFPS1b from *G. frondosa* GF9801.

Table 1
Amino acid composition of hydrolyzed GFPS1b from *G. frondosa* GF9801

Amino acid	wt%
Aspartic acid	3.32
Glutamic acid	3.01
Serine	1.53
Histidine	0.13
Glycine	1.48
Threonine	1.68
Alanine	0.37
Arginine	3.01
Tyrosine	0.36
Cystine	0.21
Valine	1.22
Methionine	0.23
Phenylalanine	0.62
Isoleucine	0.75
Leucine	1.14
Lysine	1.00
Proline	0.99

reported structure is a glucan with β-1, 6 main chains bonding attached with β-1, 3 bonding branches and trace protein (Nanba et al., 1987). This was also in accordance with the results that structures of the polysaccharides varied between the culture conditions and the purification procedures.

Data published revealed that many kinds of polysaccharides have shown significant anti-tumor activities and low side-effects in vivo (Frantz, 1989). Most reports conformed that mushroom polysaccharides exerted their anti-tumor action via activation of the immune response of the host organism, and mushroom polysaccharides were regarded as biological response modifiers (BRMs). However, in the past few years, studies related with the effects of polysaccharides on the tumor cells have increased (Lee et al., 2005; Takako et al., 2004; Li et al., 2004). It has also been reported that polysaccharides from different strains have different anti-tumor activities in vitro, depending on their monosaccharide composition, protein contents, molecular mass, and chain conformation (Jin et al., 2003). The results suggested that the polysaccharides fractions from *G. frondosa* GF9801 had selective anti-tumor activities on the different tumor cell lines (data not shown). GFPS1b had more obviously inhibitory activity on the growth of tumor cells in vitro than the other polysaccharide fractions such as GFPS0, GFPS3 and GFPS5 with the same dose and could significantly inhibit cell growth in a dose-dependent manner (Figs. 1 and 2), which was in accordance with the reports of Jin et al. (2003).

Table 2
Methylation analysis data for GFPS1b

Peak no.	Retention time	Methylated sugar derivatives	Molar ratio	Mass fragments	Linkage
1	14.06	2,3,5-Me3-Ara	1.6	43,45,71,87,101,117,129,161	Ara(1
2	14.99	2,3,5-Me3-Ara	0.1	43,45,71,87,101,117,129	Ara(1
3	18.05	2,3,6-Me3-Glu	1.2	43,45,71,87,101,113,117,129,161,173,233	4)Glu(1
4	19.24	2,4,6-Me3-Glu	2.5	43,45,71,87,101,117,129,159,161,189	3)Glu(1
5	19.56	2,3,6-Me3-Gal	1.2	43,45,87,99,101,117,129,161,233	4)Gal(1
6	21.17	2,3,4-Me3-Gal	0.5	43,44,71,87,101,117,139,130,161,189,233	6)Gal(1
7	24.17	2,4-Me2-Glu	1	43,45,71,87,99,129,189,207	1,3)Glu(6

Table 3
^{1}H NMR and ^{13}C NMR chemical shifts of polysaccharide GFPS1b

Residue	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6/C-6
Ara*f*-(α →	4.99/104.1	4.19/82.0	3.94/77.4	4.09/84.9	3.85/62.0	–
→4)Gal(1 →	5.00/96.1	3.93/69.8	3.97/69.3	4.25/78.4	4.37/69.3	3.72/61.3
→3)Glc(1 →	5.25/101.7	3.60/73.1	3.81/82.2	3.34/72.6	3.62/75.1	3.83/62.9
→1,3)Glc(6 →	5.09/103.5	3.75/74.6	3.79/85.9	3.21/69.3	3.57/76.7	3.79/70.8
→6)Gal(1 →	5.00/99.3	3.87/69.8	3.55/71.1	4.11/70.2	4.04/69.0	3.93/69.5
→4)Glc(1 →	5.08/100.0	3.64/71.6	3.91/73.7	3.71/77.3	3.79/72.0	4.01/60.9

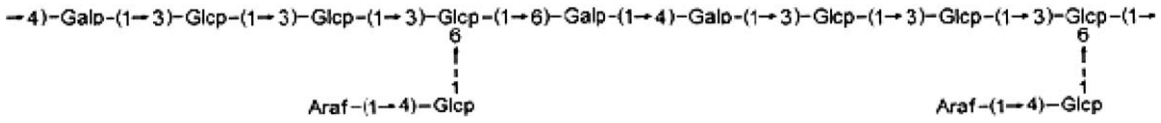

Fig. 5. Possible structure of GFPS1b.

4. Conclusion

The present study showed that the product GFPS1b from *G. frondosa* GF9801 was a novel proteo-heteroglycan with the molecular weight of 21 kDa and held the α configuration. Activity tests showed that GFPS1b was effective in the inhibition of proliferation of mammary tumor MCF-7 cells in vitro.

Acknowledgements

This work was supported by the Wuxi City Natural Sciences Foundation of China (CK030002). We would like to thank Prof. Zhongping Shi for his help in elaboration of the manuscript and Dr. Yin Li and Dr. Zhiqiang Liu for scientific suggestions.

References

Blumenkrantz, N., Asboe-Hansen, G., 1973. New method for quantitative determination of uronic acid. Anal. Biochem. 54, 484–489.

Cui, F.J., Yin, L., Xu, Z.H., Xu, H.Y., Sun, K., Tao, W.Y., in press. Optimization of the medium composition for production of mycelial biomass and *exo*-polymer by *Grifola frondosa* GF9801 using response surface methodology. Biores. Tech., doi:10.1016/j.biortech.2005.05.005.

Dubois, M., Gilles, K.A., Hamilton, J.K., Pebers, P.A., Smith, F., 1956. Colorimetric method for determination of sugar and relayed substances. Anal. Chem. 28, 350–356.

Frantz, G., 1989. Polysaccharides in pharmacy: current applications and future concepts. Planta Med. 55, 493–497.

Hakomori, S., 1964. A rapid permethylation of glycolipid and polysaccharide catalyzed by methylsulfinyl carbanion in dimethyl sulfoxide. J. Biochem. (Tokyo) 55, 205–208.

Jin, Y., Zhang, L.N., Zhang, M., Chen, L., Peter Cheung, C.K., Oi, V.E.C., Lin, Y.L., 2003. Antitumor activities of heteropolysaccharides of *Poria cocos* mycelia from different strains and culture media. Carbohydr. Res. 338, 1517–1521.

Kawagishi, H., Nomura, A., Mizuno, T., 1990. Isolation and characterization of a lectin from *Grifola frondosa* fruiting bodied. Biochim. Biophys. Acta 3, 247–252.

Kim, G.Y., Park, H.S., Nam, B.H., Lee, S.J., Lee, J.D., 2003. Purification and characterization of acidic proteo-heteroglycan from the fruiting body of *Phellinus linteus* (Berk & M.A. Curtis). Teng. Biores. Technol. 89, 81–87.

Kodama, N., Komuta, K., Sakai, N., Nanba, H., 2002. Effects of D-fraction, a polysaccharide from *Grifola frondosa* on tumor growth involve activation of NK cells. Biol. Pharm. Bull. 12, 1647–1650.

Konno, S., 2004. Potential growth inhibitory effect of Maitake D-fraction on canine cancer cells. Vet. Ther. 5, 263–271.

Lee, B.C., Bae, J.T., Pyo, H.B., Choe, T.B., Kim, S.W., Hwang, H.J., Yun, J.W., 2003. Biological activities of the polysaccharides produced from submerged culture of the edible basidiomycete *Grifola frondosa*. Enzyme Microb. Tech. 32, 574–581.

Lee, J.C., Lee, K.Y., Son, Y.O., Choi, K.C., Kim, J., Truong, T.T., Jang, Y.S., 2005. Plant-originated glycoprotein, G-120, inhibits the growth of MCF-7 cells and induces their apoptosis. Food Chem. Toxicol. 43, 961–968.

Li, G., Kim, D.H., Kim, T.D., Park, B.J., Park, H.D., Park, J.I., Na, M.K., Kim, H.C., Hong, N.D., Lin, K., Hwang, B.D., Yong, W.H., 2004. Protein-bound polysaccharide from *Phellinus linteus* induces G2/

M phase arrest and apoptosis in SW480 human colon cancer cells. Cancer Lett. 216, 175–181.

Mizuno, T., Saito, H., Nishitoba, T., Kawagishi, H., 1995. Antitumor-active substances from mushroom. Food Rev. Int. 11, 23–61.

Mondal, S., Chakraborty, I., Pramanik, M., Rout, D., Islam, S.S., 2004. Structure studies of water-soluble polysaccharide of an edible mushroom, *Termitomyces eurhizus*. A Reinvest. Carbohydr. Res. 339, 1135–1140.

Mondal, S., Chakraborty, I., Pramanik, M., Rout, D., Islam, S.S., 2005. Structural investigation of a polysaccharide (Fr. II) isolated from the aqueous extract of an edible mushroom, *Pleurotus sajor-caju*. Carbohydr. Res. 340, 629–636.

Nanba, H., 1995. Activity of Maitake D-fraction to inhibit carcinogenesis and metastasis. Cancer Prev. 786, 243–245.

Nanba, H., Hamaguchi, A., Kuroda, H., 1987. The chemical structure of an antitumor polysaccharide in fruit bodies of *Grifola frondosa* (Maitake). Chem. Pharm. Bull. (Tokyo) 35, 1162–1168.

Ohno, N., Iino, K., Taketama, T., 1985. Structure characterization and antitumor-activity of the extracts from matted mycelium of cultured *Grifola frondosa*. Chem. Pharm. Bull. 8, 3395–3401.

Suzuki, I., Itani, T., Ohno, N., 1984. Antitumor-activity of a polysaccharide fraction extracted from cultured fruiting bodied of *Grifola frondosa*. J. Pharmacobio-dynam. 7, 492–500.

Takako, S.H., Nobukazu, K., Shigeki, S., Kyogo, I., 2004. Protein-bound polysaccharide K induced apoptosis of the human Burkitt lymphoma cell line. Namalwa. Biomed. Pharmacother. 58, 226–230.

Talpur, N., Echard, B., Fan, A., 2002. Antihypertensive and metabolic effects of whole Maitake mushroom powder and its fractions in two rat strains. Mol. Cell Biochem. 237, 129–136.

Wang, X.S., Liu, L., Fang, J.N., 2005. Immunological activities and structure of pectin from *Centella asiatica*. Carbohydr. Polym. 60, 95–101.

Zhuang, C., Mizuno, T., Ito, H., 1994. Antitumor-activity and immunological property of polysaccharides from the mycelium of liquid cultured *Grifola frondosa*. J. Jpn. Soc. Food. Sci. 10, 724–732.