Carbohydrate Polymers 88 (2012) 474–480

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

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

Purification, characterization and anti-proliferation activity of polysaccharides from *Flammulina velutipes*

Wenjian Yang[a,b], Fei Pei[b], Ying Shi[c], Liyan Zhao[b], Yong Fang[a], Qiuhui Hu[a,b,*]

[a] College of Food Science and Engineering, Nanjing University of Finance and Economics, Nanjing, 210046, China
[b] College of Food Science and Technology, Nanjing Agricultural University, Weigang, Nanjing 210095, China
[c] College of Science, Nanjing Agricultural University, Weigang, Nanjing 210095, China

ARTICLE INFO

Article history:
Received 15 October 2011
Received in revised form
15 November 2011
Accepted 13 December 2011
Available online 21 December 2011

Keywords:
Flammulina velutipes
Polysaccharide
Isolation
Characterization
Anti-proliferation activity

ABSTRACT

A polysaccharide was isolated from *Flammulina velutipes* (FVP) using ultrasonic-assisted extraction, and then further purified by DEAE cellulose-52 chromatography and Sephadex G-100 size-exclusion chromatography to afford FVP-1 and FVP-2. Structural characteristics of FVP-1 and FVP-2 (including molecular weight, monosaccharides composition, sulfate and uronic acid contents, triple helical structures, ultraviolet spectrum, and infrared spectrum) were investigated, and their anti-proliferation activities against human gastric cancer BGC-823 cells and lung cancer A549 cells were researched *in vitro*. Results suggested that both FVP-1 and FVP-2 could significantly suppress the proliferation of BGC-823 cells in a concentration-dependent manner. The highest inhibitory rates were 78% for FVP-1 and 95% for FVP-2 at the concentration of 200 μg/ml, respectively. This high anti-proliferation activity was inferred to be owing to the structural characterizations of polysaccharide, and the recognition of biological system for the triple helical structures of polysaccharides.

1. Introduction

For thousands of years, mushrooms have been known as an important source of nutritional diet and medicine. Extensive studies have revealed that many species of mushrooms had promising potential in improving human health and preventing diseases (Mattila, Suonpää, & Piironen, 2000). Polysaccharides, as one of the most important components isolated from mushrooms, have been correlated with multiple pharmacological activities, such as antioxidant, immunomodulatory, reducing blood lipid, and anti-tumor activities (Liu, Ooi, & Chang, 1997; Wasser, 2002; Zhang, Cui, Cheung, & Wang, 2007).

Flammulina velutipes, one of the most popular edible mushrooms, has being under a large-scale artificial cultivation and increasingly consumed in Japan and China owing to its high nutritional values and attractive taste. Therefore, much attention has been paid on the active constituents and pharmacological properties of *F. velutipes*. Several polysaccharides have been isolated from *F. velutipes* fruit bodies or mycelium, and their pharmacological efficacies were investigated. For example, an anti-tumor polysaccharide with the structure of β-1,3-glucan was isolated from cytoplasms of the fruit bodies of *F. velutipes* (Ikekawa et al., 1982). An alkaline-soluble polysaccharide prepared from the cell wall of *F. velutipes* was reported to have immunomodulatory and anti-tumor activities against sarcoma SC180 (Leung, Fung, & Choy, 1997). In addition, a polysaccharide isolated from *F. velutipes* was proved to exert anti-inflammatory effect by decreasing $CD4^+$, $CD8^+$, ICAM-1, and MPO levels in serum and colon of burned rats (Wu, Duan, Liu, & Cen, 2010). Up to date, various methods are applied to increase the extraction efficiency of polysaccharides from mushrooms, including selection of proper solvents, prolonging extraction time, heating process, and agitation. However, these strategies are generally time- and high-energy consuming, and low yield. Recently, Ultrasonic wave has been widely utilized for the extraction of bioactive components from natural products aimed to improve their extraction efficiency. However, there are few investigations focusing on the ultrasonic-assisted extraction of polysaccharides from *F. velutipes* (FVP), and on the effect of FVP on the proliferation of human gastric cancer cells (BGC-823) and lung cancer cells (A549). Moreover, limited information is available on the structure–activity relationships of polysaccharides.

In our previous study, ultrasonic-assisted extraction was performed for preparation of FVP, and its yiled was increased by 61.9% compared with hot-water extraction (Yang, Fang, Liang, & Hu, 2011). As a part of our ongoing project, the FVP prepared previously in our lab was further isolated with DEAE-52 cellulose chromatography and Sephadex G-100 chromatography, and their effects on proliferation was investigated in human gastric cancer

* Corresponding author at: College of Food Science and Engineering, Nanjing University of Finance and Economics, Nanjing, 210046, China. Tel.: +86 25 84399086; fax: +86 25 84399086.
E-mail address: qiuhuihu@njau.edu.cn (Q. Hu).

0144-8617/$ – see front matter.
doi:10.1016/j.carbpol.2011.12.018

BGC-823 cells and lung cancer A549 cells. Meantime, to well understand the structure–activity relationship of FVPs, their molecular weight, monosaccharide composition, sulfate and uronic acid contents, triple helical structures were analyzed as well.

2. Materials and methods

2.1. Materials and chemicals

F. velutipes was purchased from a local market (Nanjing, China). Human gastric cancer cells (BGC-823) and lung cancer cells (A549) were obtained from Nanjing University state key lab of pharmaceutical biotechnology (Nanjing, China). DEAE-cellulose 52 and Sephadex G-100 were purchased from Whatman Co. (Maidstone, Kent, UK) and Pharmacia Co. (Sweden), respectively. Rhamnose, arabinose, fucose, xylose, mannose, glucose, galactose, inositol, dimethylsulfoxide (DMSO), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were from Sigma Chemical Co. (St. Louis, MO, USA). T-series dextrans were purchased from Amersham Pharmacia (Uppsala, Sweden). Dulbecco's minimal essential medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were purchased from Invitrogen (Carlsbad, CA, USA). All other reagents were of analytical grade.

2.2. Preparation of FVP

Fresh *F. velutipes* was dried at 60 °C, powdered and sieved through a No. 300 mesh. The *F. velutipes* powder was extracted with distilled water by ultrasonic treatment in ultrasonic cell disintegrator (DCTZ-2000, Beijing Hongxianglong Biotechnology Development Co. Ltd.), and the extraction was carried out under an optimized condition (ratio of water to material of 25 ml/g, ultrasonic power of 620 W, ultrasonic time of 20 min, and ultrasonic temperature of 45 °C) according to our previous study (Yang et al., 2011). Crude polysaccharides extract was deproteinized with Sevag reagent (chloroform:butanol, 4:1), and then precipitated with 4-fold volume anhydrous ethanol. After centrifugation at 5000 rpm/min for 15 min, the precipitate was washed successively with anhydrous ethanol and acetone, dialyzed against deionized water, and lyophilized as crude polysaccharides (FVP).

2.3. Isolation and purification of FVP

FVP (150 mg) was dissolved in distilled water, and then filtered through 0.45 μm membrane. The FVP solution was subjected to a DEAE-52 cellulose column (2.6 cm × 50 cm) with a stepwise elution of NaCl solution (0, 0.1, 0.3, and 0.5 M). The eluates were combined according to the total carbohydrate content quantified by phenol–sulfuric acid method to afford F-1, F-2 and F-3. After dialyzing and concentrating, F-1 and F-2 were further purified with a Sephadex G-100 column (2.6 cm × 60 cm). Distilled water served as the eluants at a flow rate of 0.25 ml/min. Finally, FVP-1 and FVP-2 were obtained from F-1 and F-2, respectively.

2.4. Molecular weights of FVP-1 and FVP-2

The average molecular weights of FVP-1 and FVP-2 were determined by high-performance size-exclusion chromatography (HPSEC) on an Agilent 1200 system equipped with a TSK gel G4000 PW_{XL} column (300 mm × 7.8 mm) and an evaporative light scattering detector (ELSD) (Zhu et al., 2010). Twenty microliter sample solution was injected and eluted with distilled water at a flow rate of 0.7 ml/min. The linear regression was calibrated with T-series dextrans standards (T-500, T-200, T-100, T-50, and T-10).

2.5. Monosaccharides composition

Monosaccharides composition of FVP-1 and FVP-2 were analyzed by gas chromatography (GC) as described by Sheng et al. (2007) with some slight modifications. Briefly, 5 g of FVP-1 or FVP-2 was hydrolyzed with 4 ml 2 mol/l of trifluoroactic acid (TFA) at 110 °C for 2 h. After removing the residual TFA with methanol under reduced pressure, the sample was dissolved in 0.6 ml of pyridine and reacted with 10 mg of hydroxylamine hydrochloride and 2 mg of inositol (as internal reference) for 30 min at 90 °C. Afterwards, 0.8 ml of acetic anhydride was added and incubated for anther 30 min at 90 °C. Eight standard sugars (rhamnose, arabinose, xylose, mannose, glucose, fructose, fucose, and galactose) were converted to their acetylated derivatives according to the above-mentioned method.

One microliter of sample derivatives was injected into Agilent 6890 N GC equipped with a HP-5 fused silica capillary column (30 m × 0.32 mm × 0.25 mm) and a flame ionization detector (FID). The oven temperature was maintained at 120 °C for 3 min, and then increased gradually to 210 °C at a rate of 3 °C/min. The relative molar proportions of sugars in polysaccharide were calculated by the area normalization method according to the chromatogram.

2.6. Determination of sulfate, and uronic acid contents

The content of sulfate groups in polysaccharide was measured by the barium chloride–gelatin method (Dodgson & Price, 1962). FVP-1 or FVP-2 was hydrolyzed with 1 mol/l hydrochloric acid, and then dried under reduced pressure. The hydrolysate was dissolved with 1 ml of water and reacted with 8% of trichloroacetic acid and 5% of barium chloride–gelatin solution for 20 min. The absorbance of the reaction solution was determined at 360 nm. Gelatin solution was used instead of barium chloride–gelatin solution for elimination of interference. A series of concentrations of potassium sulfate solution (0, 20, 40, 60, 80, and 100 μg/ml) were used to obtain a standard curve.

The content of uronic acid in polysaccharide was determined using sulfuric acid-carbazole method (Karamanos, Hjerpe, Tsegenidis, Engfeldt, & Antonopoulos, 1988). One milliliter of sample reacted with 5 ml 9.54 mg/ml of sodium tetraborate sulfuric acid solution at boiling water bath for 10 min, and then mixed with 0.2 ml 1.25 mg/ml of carbazole ethanol solution for another 10 min. The absorbance of the reaction solution was determined at 530 nm. D-Glucuronic acid was served as the reference.

2.7. Triple helical structures of FVP-1 and FVP-2

The triple helical structures of FVP-1 and FVP-2 were analyzed by the interaction with Congo red as previously described (Ogawa, Wanatabe, Tsurugi, & Ono, 1972; Rout, Mondal, Chakraborty, & Islam, 2008). Briefly, 5 mg of polysaccharide was dissolved in 2.0 ml of distilled water and reacted with 2.0 ml 80 μmol/l of Congo red in a gradient of sodium hydroxide solutions (0.0, 0.1, 0.2, 0.3, 0.4, and 0.5 mol/l). The absorbance was measured in the range of 200–800 nm, and the maximum absorption wavelength (λ_{max}) at different concentrations of sodium hydroxide was plotted. Distilled water without adding polysaccharide was served as the control.

2.8. Ultraviolet (UV) and Fourier-transform infrared spectra (FT-IR) analysis

Ultraviolet and infrared spectra were performed as previous descriptions (Zhao, Dong, Chen, & Hu, 2010) The ultraviolet spectra of FVP-1 and FVP-2 solutions were determined with an ultraviolet spectrophotometer (Shanghai Precision and Scientific Instrument Co. Ltd., Shanghai, China) in the range of 200–400 nm. FT-IR

spectra of FVP-1 and FVP-2 were determined using the KBr-disks method and scanned in the range of 500–4000 cm^{-1} with a Nicolet Fourier transform infrared spectrometer (NICOLET NEXUS470, Thermo Nicolet Co., WI, USA).

2.9. Anti-proliferation assay

Human gastric cancer BGC-823 and lung cancer A549 cells were incubated in DMEM supplemented with 10% FBS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin at 37 °C in a humidified atmosphere of 5% CO_2.

Anti-proliferation assay were performed using MTT method as previously described with some modifications (Kassim, Achoui, Mustafa, Mohd, & Yusoff, 2010). Briefly, 100 μl of cells suspension at a density of 5×10^5 cells/ml was seeded in 96-well plates for 12 h incubation, and then the cells were treated with the medium containing different concentrations of polysaccharides (FVP-1 or FVP-2) for 24 h. After removing the medium, 10 μl of MTT solution was added to each well and incubated for another 4 h. Subsequently, 100 μl of DMSO was added to dissolve formazan crystals. Absorbance was measured at 570 nm with a microplate reader (Thermo multiskan Mk3, Thermo Fisher Scientific Inc., USA).

2.10. Statistical analysis

All experiments were carried out in triplicate and data were expressed as mean ± standard deviation. Statistical analysis was performed with one-way analysis of variance (ANOVA). Values of $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. Isolation and purification of crude FVP

Crude FVP was further isolated by an anion-exchange chromatography column of DEAE-52 cellulose based on the difference of ionic groups present in FVP molecule. Three fractions eluted with 0, 0.1, 0.3 M sodium chloride solutions were collected (F-1, F-2, and F-3), respectively (Fig. 1A). F-1 eluted with deionized water was known as neutral polysaccharides, and F-2 and F-3 eluted with 0.1 M and 0.3 M NaCl were known as acidic polysaccharides (Gan, Ma, Jiang, Xu, & Zeng, 2011). Considering the yield of three factions (F-3 just accounted for 5% of the total), two major fractions (F-1 and F-2) were further purified by gel-filtration on Sephadex G-100, and then concentrated, dialyzed and lyophilized to afford FVP-1 and FVP-2 (Fig. 1B and C).

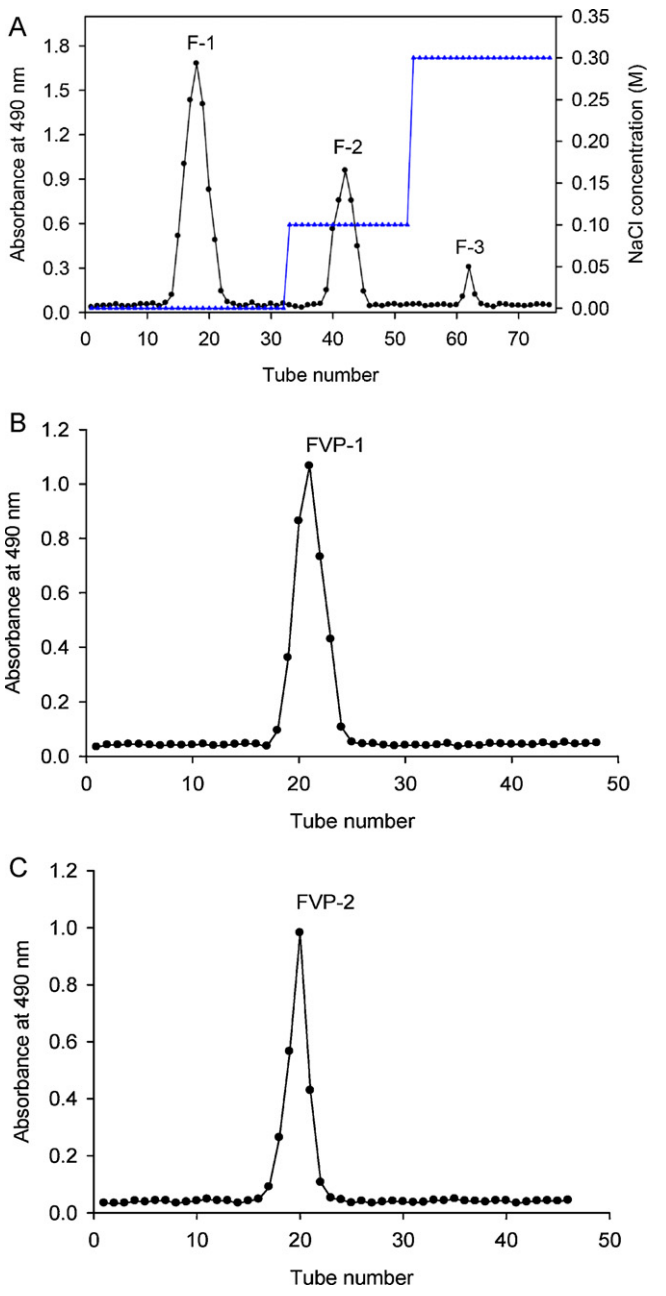

Fig. 1. (A) Elution profile of FVP on DEAE-52 chromatography column with gradient of NaCl solution (0, 0.1, and 0.3 M); (B) elution profile of F-1 on Sephadex G-100 gel chromatography column with distilled water; (C) elution profile of F-2 on Sephadex G-100 gel chromatography column with distilled water.

3.2. Molecular weight, monosaccharides composition, sulfate, and uronic acid contents of FVP-1 and FVP-2

Both FVP-1 and FVP-2 were shown as single symmetrical peak in their respective HPSEC chromatograms, indicating that they were homogeneous (Fig. 2). The relative molecular weights of FVP-1 and FVP-2 were 28 kDa and 268 kDa, respectively (Table 1). Chromatograms of monosaccharides composition of FVP-1 and FVP-2 were shown in Fig. 3, and the molar ratio of sugars were summarized in Table 1. FVP-1 was composed of glucose, galactose, mannose and fucose in an approximate molar ratio 81.3:12.1:3.6:3.0, and FVP-2 was composed of glucose, mannose, galactose, xylose and fucose in an approximate molar ratio 57.9:12.0:15.1:9.5:5.5. This suggested that both FVP-1 and

Table 1
Preliminary characteristics of FVP-1, and FVP-2.

Sample	Molecular weight (kDa)	Uronic acid (%)	Sulfate (%)	Monosaccharides composition (%)[a]							
				Fru	Glu	Ara	Fuc	Xyl	Man	Rha	Gal
FVP-1	28	1.56	0.09	–[b]	81.3	–	3.0	–	3.6	–	12.1
FVP-2	268	3.42	0.14	–	57.9	–	5.5	9.5	15.1	–	12.0

[a] Individual components were identified and quantified based on elution of known standards, and data are presented as mol% for each sugar.
[b] Not detected.

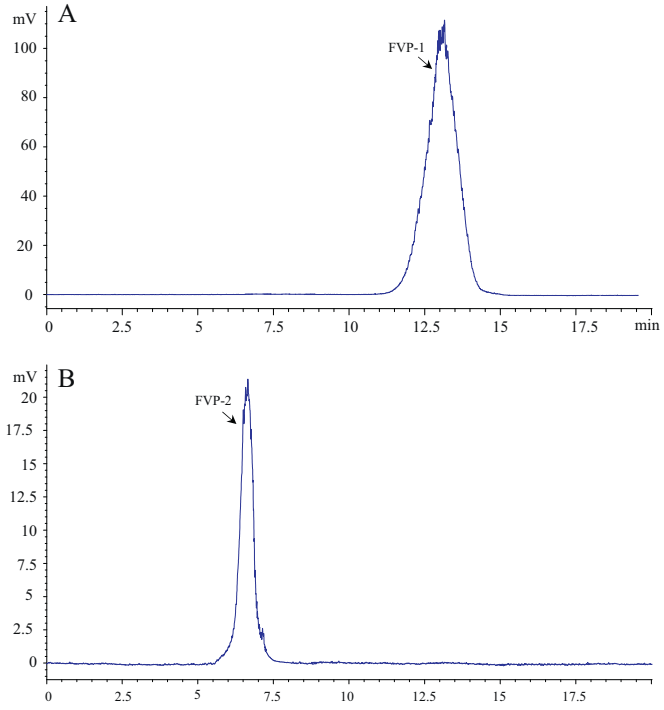

Fig. 2. High-performance size-exclusion chromatography (HPSEC) of FVP-1 (A) and FVP-2 (B).

FVP-2 were heteropolysaccharides with a glucan as backbone chain. Sulfate contents of FVP-1 and FVP-2 were 0.09% and 0.14%, respectively, and uronic acid contents of FVP-1 and FVP-2 were 1.56% and 3.42%, respectively (Table 1).

3.3. Triple helical structures of FVP-1 and FVP-2

The interactions of FVP-1 and FVP-2 with Congo red were shown in Fig. 4. The maximum absorption wavelength (λ_{max}) of Congo red was largely shifted in the presence of FVP-1 or FVP-2 (increased initially and then reduced gradually to reach constant with the increasing NaOH concentration), which indicated that both FVP-1 and FVP-2 had triple-helix conformation, and the triple helical structure was destroyed in high concentrate of NaOH solution (Rout et al., 2008).

3.4. Ultraviolet and infrared spectra of FVP-1 and FVP-2

No absorption at 280 nm and 260 nm were observed for both FVP-1 and FVP-2 in the UV spectra (Fig. 5), indicating the absence of protein and nucleic acid. The infrared spectra of FVP-1 and FVP-2 ranged from 500 cm^{-1} to 4000 cm^{-1} were shown in Fig. 6. A broadly stretched intense peak at 3400 cm^{-1} was regarded as the characteristic absorption of hydroxyl groups (—OH) (Zhao et al., 2010). The peaks at around 2926 cm^{-1} and 2360 cm^{-1} were the characteristic absorptions of C—H and aliphatic C—H bonds, respectively (Yang et al., 2006). The peaks at 2340 cm^{-1} and 1653 cm^{-1} indicated that there would be —C≡N and N—H groups in FVP-1 and FVP-2, respectively (Gan et al., 2011; Ikhuoris, Folayan, & Okieimen, 2010).

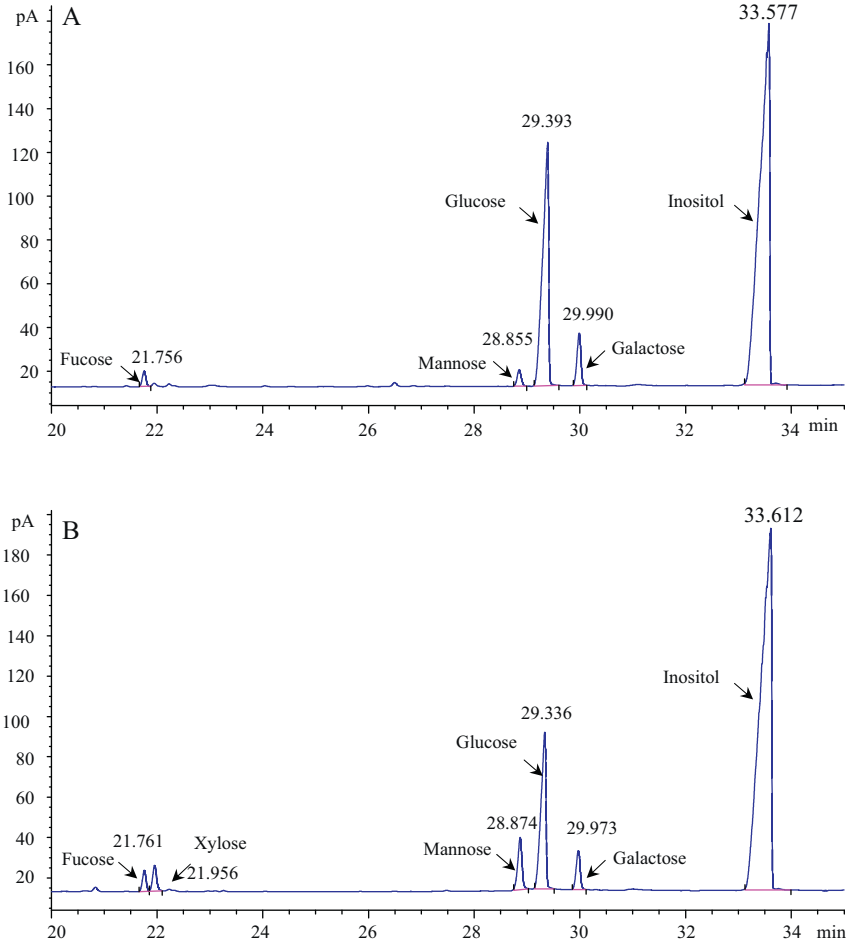

Fig. 3. GC chromatograms of monosaccharides composition of FVP-1 (A) and FVP-2 (B) with a HP-5 fused silica capillary column.

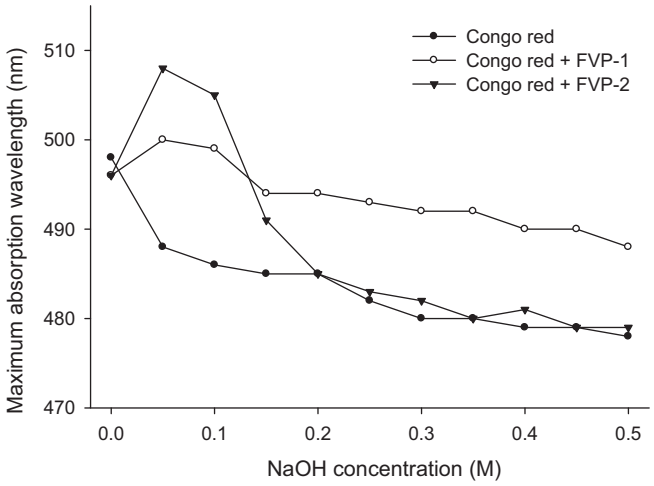

Fig. 4. The maximum absorption wavelength (λ_{max}) of Congo red in the presence of FVP-1 and FVP-2 at various concentrations of sodium hydroxide solution.

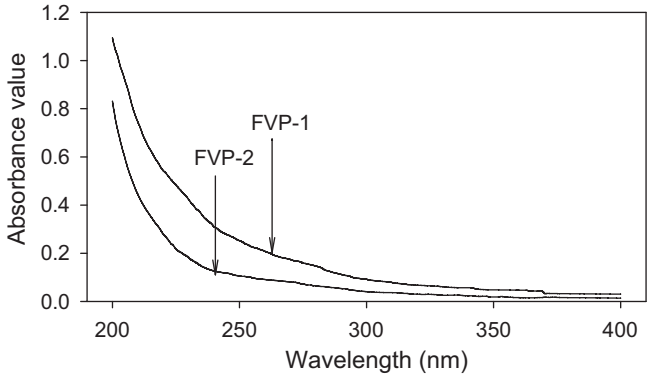

Fig. 5. UV spectra of FVP-1 and FVP-2 in the range of 200–400 nm.

The absorbance of the asymmetric stretching of ester sulfate band (S=O) at 1250 cm^{-1} was used to calculate total ester sulfate content of polysaccharide (Melo, Feitosa, Freitas, & de Paula, 2002), and the ratio of absorbance of 1250/2920 cm^{-1} was used to determine the degree of substitution with sulfate of disaccharide repeat unit (Rochas, Lahaye, & Yaphe, 1986). Three strong absorption peaks of FVP-1 and two strong absorption peaks of FVP-2 within the range of 1100–1010 cm^{-1} indicated the possible presence of pyranose ring in FVP-1 and furanose ring in FVP-2, respectively (Qiao et al., 2010). A diagnostic absorption peak at about 892 cm^{-1} suggested that the glycosyl residues of FVP-1 and FVP-2 were mainly β-type glycosidic linkages (Fariña, Viñarta, Cattaneo, & Figueroa, 2009).

3.5. Anti-proliferation assay

Human gastric cancer cells (BGC-823) and lung cancer cells (A549) were employed to investigate the anti-proliferation activities of FVP-1 and FVP-2. As shown in Fig. 7A, no significant

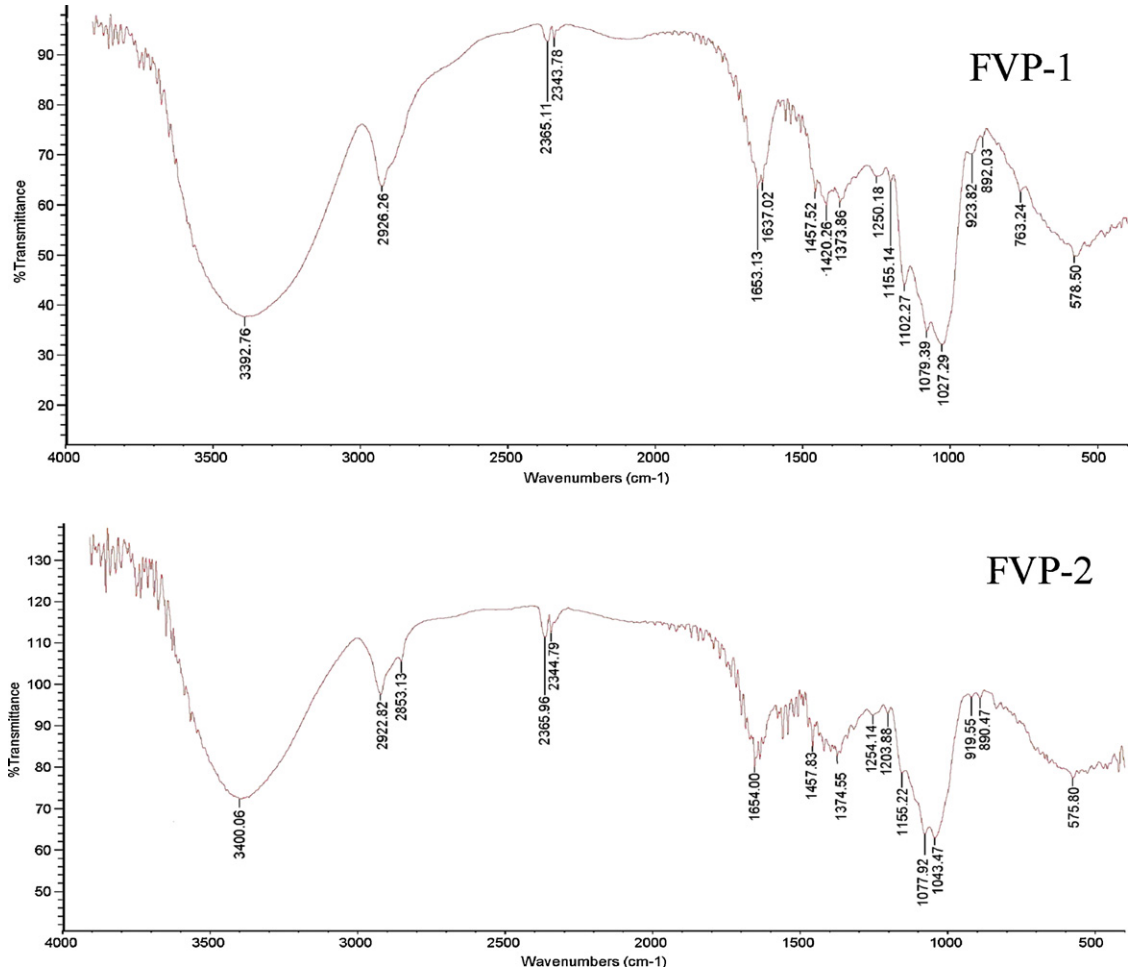

Fig. 6. FT-IR spectra of FVP-1 and FVP-2 in the range of 400–4000 cm^{-1}.

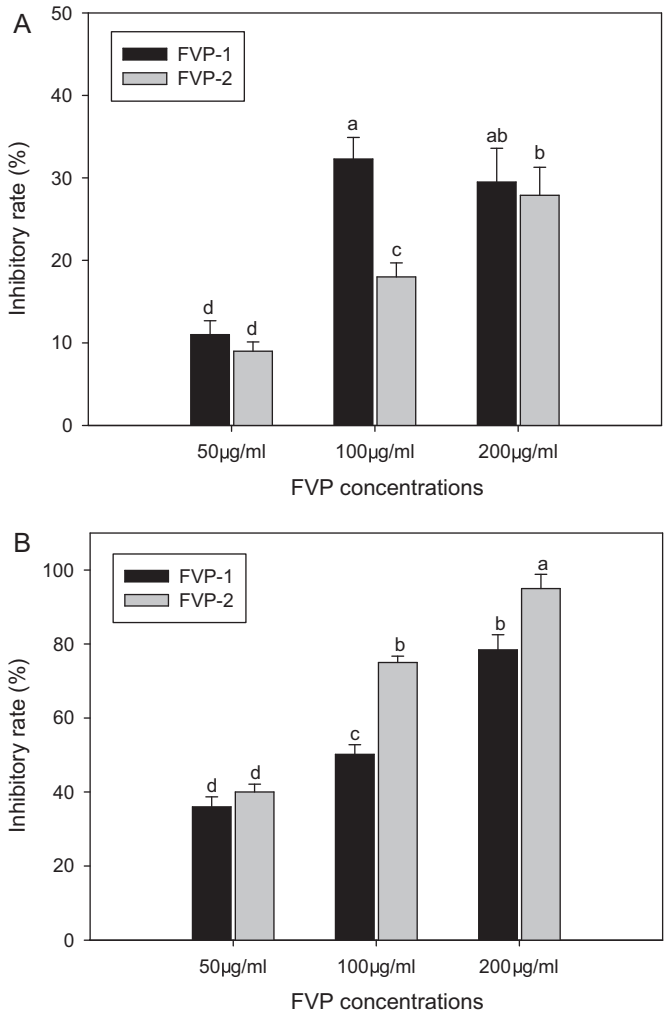

Fig. 7. Anti-proliferation activities of different concentrations of FVP-1 and FVP-2 fractions against A549 cells (A) and BGC-823 cells (B). Values are means ± S.D. of three determinations. Statistically significant differences at P values of <0.05.

difference was observed for the inhibitory rate of A549 cells between FVP-1 and FVP-2 at 50 μg/ml and 200 μg/ml. FVP-1 at 100 μg/ml exhibited the strongest anti-proliferation activtiy against A549 cells with an inhibitory rate of 32.3%, significantly higher compared with FVP-2 at 200 μg/ml (27.9%) ($P < 0.05$). As for BGC-823 cells, both FVP-1 and FVP-2 could significantly suppress the proliferation of cells in a concentration-dependent manner (Fig. 7B). The highest inhibitory rates were 78% for FVP-1 and 95% for FVP-2 at the concentration of 200 μg/ml, respectively. At 100 μg/ml, FVP-2 also exhibited the more potent anti-proliferation effect on BGC-823 cells than FVP-1 ($P < 0.05$).

4. Discussion

Multiple pharmacological properties of polysaccharides have been revealed, and these activities are correlated with their chemical composition and configuration and physical properties. Although it is difficult to clarify the structure–activity correlation of complex polysaccharides, some possible relationships can be inferred. Acidic polysaccharides were thought to be more biologically active than neutral polysaccharides (Gan et al., 2011), and polysaccharides with higher sulfate and uronic acid contents presented stronger antioxidant activities and higher inhibitory effect on the growth of human gastric cancer BGC-823 cells *in vitro* (Jiang, Wang, Liu, Gan, & Zeng, 2011; Qi et al., 2005). In this study, the acidic polysaccharide FVP-2 exhibited higher inhibitory activity against gastric cancer cells than neutral polysaccharide FVP-1, which might be attributed to the presence of more sulfate and uronic acid in FVP-2.

Molecular weight and monosaccharides composition were also regarded as two important factors related to the anti-cancer activities of polysaccharides. Chen, Xu, Zhang, and Zeng (2009) found that polysaccharides with relatively high molecular weight exhibited stronger inhibitory activity against S-180 tumor cells. Ooi and Liu (2000) reported that mushroom polysaccharides with high anti-tumor activities were mostly heteropolysaccharides and had a monosaccharides composition of galactose, glucose, mannose and fucose. Our results were in good agreement with the above reports. The molecular weights of FVP-1 and FVP-2 were approximately 28 kDa and 268 kDa, respectively. Monosaccharides composition analysis demonstrated that galactose, glucose, mannose, and fucose were present in both FVP-1 and FVP-2, and 5.5% xylose also participated in FVP-2. These structural features may be responsible for the higher anti-proliferation activity of FVP-2 than FVP-1.

In addition, triple helical structure of polysaccharides might be related to their pharmacological activities. Lentinan with triple helix structure was demonstrated a relatively higher inhibition ratio against Sarcoma 180 tumor than that with single flexible structure (Zhang, Li, Xu, & Zeng, 2005), this antitumor activity apparently depended on the recognition of biological system for the helical structure of polysaccharides (Ooi & Liu, 2000). In the present study, both FVP-1 and FVP-2 have triple helix structure, and excellent inhibitory activity against gastric cancer cells rather than against lung cancer cells was found for FVP-1 and FVP-2. These results also indicated the recognition of biological system for polysaccharides, as well as the targeting selection of polysaccharides for cancer cells. Nevertheless, to thoroughly elucidate the structure–activity relationship of polysaccharides, all structural characteristics should be comprehensively taken into account. Meantime, more effort should be made to well understand the structure–activity of polysaccharides in the future.

5. Conclusions

In this study, FVP was extracted using ultrasonic wave and isolated by DEAE-52 and Sephadex G-100 to afford FVP-1 and FVP-2, with the molecular weights of 28 kDa and 268 kDa, respectively. FVP-1 and FVP-2 exhibited prominent inhibitory activities against BGC-82 cells rather than against A549 cells, which could be related to their molecular weight, monosaccharides composition, sulfate, and uronic acid contents, and triple helical structures of polysaccharides. These results suggest the potential prospects of FVP as functional ingredient to prevent gastric cancer.

Acknowledgement

This work is financially supported by the earmarked fund for Modern Agro-industry Technology Research System (CARS-24).

References

Chen, X., Xu, X., Zhang, L., & Zeng, F. (2009). Chain conformation and anti-tumor activities of phosphorylated (1 → 3)-[beta]-D-glucan from Poria cocos. *Carbohydrate Polymers*, *78*, 581–587.

Dodgson, K. S., & Price, R. G. (1962). A note on the determination of the ester sulphate content of sulphated polysaccharides. *Biochemical Journal*, *84*, 106–110.

Fariña, J. I., Viñarta, S. C., Cattaneo, M., & Figueroa, L. I. C. (2009). Structural stability of *Sclerotium rolfsii* ATCC 201126 β-glucan with fermentation time: a chemical, infrared spectroscopic and enzymatic approach. *Journal of Applied Microbiology*, *106*, 221–232.

Gan, D., Ma, L., Jiang, C., Xu, R., & Zeng, X. (2011). Production, preliminary characterization and antitumor activity in vitro of polysaccharides from the mycelium of *Pholiota dinghuensis* Bi. *Carbohydrate Polymers*, *84*, 997–1003.

Ikekawa, T., Ikeda, Y., Yoshioka, Y., Nakanishi, K., Yokoyama, E., & Yamazaki, E. (1982). Studies on antitumor polysaccharides of *Flammulina velutipes* (Curt. ex Fr.) Sing. II. The structure of EA3 and further purification of EA5. *Journal of Pharmacobiodynamics*, *5*, 576–581.

Ikhuoris, E. U., Folayan, A. S., & Okieimen, F. E. (2010). Studies in the graft copolymerization of acrylonitrile onto cassava starch by ceric ion induced initiation. *International Journal for Biotechnology and Molecular Biology Research*, *1*, 010–014.

Jiang, C., Wang, M., Liu, J., Gan, D., & Zeng, X. (2011). Extraction, preliminary characterization, antioxidant and anticancer activities in vitro of polysaccharides from *Cyclina sinensis*. *Carbohydrate Polymers*, *84*, 851–857.

Karamanos, N. K., Hjerpe, A., Tsegenidis, T., Engfeldt, B., & Antonopoulos, C. A. (1988). Determination of iduronic acid and glucuronic acid in glycosaminoglycans after stoichiometric reduction and depolymerization using high-performance liquid chromatography and ultraviolet detection. *Analytical Biochemistry*, *172*, 410–419.

Kassim, M., Achoui, M., Mustafa, M. R., Mohd, M. A., & Yusoff, K. M. (2010). Ellagic acid, phenolic acids, and flavonoids in Malaysian honey extracts demonstrate in vitro anti-inflammatory activity. *Nutrition Research*, *30*, 650–659.

Leung, M. Y. K., Fung, K. P., & Choy, Y. M. (1997). The isolation and characterization of an immunomodulatory and anti-tumor polysaccharide preparation from *Flammulina velutipes*. *Immunopharmacology*, *35*, 255–263.

Liu, F., Ooi, V. E. C., & Chang, S. T. (1997). Free radical scavenging activities of mushroom polysaccharide extracts. *Life Sciences*, *60*, 763–771.

Mattila, P., Suonpää, K., & Piironen, V. (2000). Functional properties of edible mushrooms. *Nutrition*, *16*, 694–696.

Melo, M. R. S., Feitosa, J. P. A., Freitas, A. L. P., & de Paula, R. C. M. (2002). Isolation and characterization of soluble sulfated polysaccharide from the red seaweed *Gracilaria cornea*. *Carbohydrate Polymers*, *49*, 491–498.

Ogawa, K., Wanatabe, T., Tsurugi, J., & Ono, S. (1972). Conformational behavior of a gel-forming (1 → 3)-β-glucan in alkaline solution. *Carbohydrate Research*, *23*, 399–405.

Ooi, V. E., & Liu, F. (2000). Immunomodulation and anti-cancer activity of polysaccharide–protein complexes. *Current Medicinal Chemistry*, *7*, 715–729.

Qi, H., Zhang, Q., Zhao, T., Chen, R., Zhang, H., Niu, X., et al. (2005). Antioxidant activity of different sulfate content derivatives of polysaccharide extracted from *Ulva pertusa* (Chlorophyta) in vitro. *International Journal of Biological Macromolecules*, *37*, 195–199.

Qiao, D., Liu, J., Ke, C., Sun, Y., Ye, H., & Zeng, X. (2010). Structural characterization of polysaccharides from *Hyriopsis cumingii*. *Carbohydrate Polymers*, *82*, 1184–1190.

Rochas, C., Lahaye, M., & Yaphe, W. (1986). Sulfate content of carrageenan and agar determined by infrared spectroscopy. *Botanica Marina*, *29*, 335–340.

Rout, D., Mondal, S., Chakraborty, I., & Islam, S. S. (2008). The structure and conformation of a water-insoluble (1 → 3)-,(1 → 6)-[beta]-D-glucan from the fruiting bodies of *Pleurotus florida*. *Carbohydrate Research*, *343*, 982–987.

Sheng, J., Yu, F., Xin, Z., Zhao, L., Zhu, X., & Hu, Q. (2007). Preparation, identification and their antitumor activities in vitro of polysaccharides from *Chlorella pyrenoidosa*. *Food Chemistry*, *105*, 533–539.

Wasser, S. P. (2002). Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. *Applied Microbiology and Biotechnology*, *60*, 258–274.

Wu, D., Duan, W., Liu, Y., & Cen, Y. (2010). Anti-inflammatory effect of the polysaccharides of Golden needle mushroom in burned rats. *International Journal of Biological Macromolecules*, *46*, 100–103.

Yang, B., Wang, J., Zhao, M., Liu, Y., Wang, W., & Jiang, Y. (2006). Identification of polysaccharides from pericarp tissues of litchi (*Litchi chinensis* Sonn.) fruit in relation to their antioxidant activities. *Carbohydrate Research*, *341*, 634–638.

Yang, W., Fang, Y., Liang, J., & Hu, Q. (2011). Optimization of ultrasonic extraction of *Flammulina velutipes* polysaccharides and evaluation of its acetylcholinesterase inhibitory activity. *Food Research International*, *44*, 1269–1275.

Zhang, L., Li, X., Xu, X., & Zeng, F. (2005). Correlation between antitumor activity, molecular weight, and conformation of lentinan. *Carbohydrate Research*, *340*, 1515–1521.

Zhang, M., Cui, S. W., Cheung, P. C. K., & Wang, Q. (2007). Antitumor polysaccharides from mushrooms: a review on their isolation process, structural characteristics and antitumor activity. *Trends in Food Science and Technology*, *18*, 4–19.

Zhao, L., Dong, Y., Chen, G., & Hu, Q. (2010). Extraction, purification, characterization and antitumor activity of polysaccharides from *Ganoderma lucidum*. *Carbohydrate Polymers*, *80*, 783–789.

Zhu, B., Zhou, D., Li, T., Yan, S., Yang, J., Li, D., et al. (2010). Chemical composition and free radical scavenging activities of a sulphated polysaccharide extracted from abalone gonad (Haliotis Discus Hannai Ino). *Food Chemistry*, *121*, 712–718.