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Isolation of a polysaccharide with anticancer activity from *Auricularia polytricha* using high-speed countercurrent chromatography with an aqueous two-phase system

Guanglei Song, Qizhen Du*

Institute of Food and Biological Engineering, Hangzhou University of Commerce, 149 Jiaogong Road, Hangzhou, Zhejiang 310035, China

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ABSTRACT

Polysaccharides from a crude extract of *Auricularia polytricha* were separated by high-speed countercurrent chromatography (HSCCC). The separation was performed with an aqueous two-phase system of PEG1000–K_2HPO_4–KH_2PO_4–H_2O (0.5:1.25:1.25:7.0, w/w). The crude sample (2.0 g) was successfully separated into three polysaccharide components of AAPS-1 (192 mg), AAPS-2 (137 mg), and AAPS-3 (98 mg) with molecular weights of 162, 259, and 483 kDa, respectively. These compounds were tested for growth inhibition of transplanted S180 sarcoma in mice. AAPS-2 had an inhibition rate of 40.4%. The structure of AAPS-2 was elucidated from partial hydrolysis, periodate oxidation, acetylation, methylation analysis, and NMR spectroscopy (^1H, ^{13}C). These results showed AAPS-2 is a polysaccharide with a backbone of (1 → 3)-linked-β-D-glucopyranosyl and (1 → 3, 6)-linked-β-D-glucopyranosyl residues in a 2:1 ratio, and has one terminal (1→)-β-D-glucopyranosyl at the O-6 position of (1→3, 6)-linked-β-D-glucopyranosyl of the main chain.

1. Introduction

Due to their interesting biological activities, mushrooms have recently become an attractive source material for the development of pharmaceutical products [1]. Many polysaccharides have been isolated from mushrooms, fungi, yeast, algae, lichens, and plants in recent years, and screened for biological activity [2,3]. Most polysaccharides derived from plants are relatively nontoxic and do not cause significant side effects. These could allow development of an effective natural anticancer with few side effects. *Auricularia polytricha*, which belongs to the Auriculariaceae family, is widespread in many districts of China. Modern pharmacology research indicates that it has antioxidant, antitumor, and immunomodulatory activities [4–6]. Lentinan, a polysaccharide from the Shiitake mushroom (*Lentinula edodes*), has been demonstrated to have anticancer activity [7–9]. This inspired us to investigate the potential anticancer activity of *A. polytricha*. In this study, polysaccharides were isolated from *A. polytricha* using high-speed countercurrent chromatography (HSCCC) and an aqueous two-phase system. These systems have been successfully applied as the solvent in HSCCC to separate bioactive macromolecules such as polysaccharides and proteins [10,11].

2. Experimental

2.1. Reagents and materials

All chemical reagents were of analytical grade, and were purchased from Huadong Chemicals (Hangzhou, China). The polyethylene glycol (PEG) 1000 used had an average molecular weight of 950–1050 Da. Fresh *A. polytricha* were purchased at a local store in Hangzhou, China. Papain was provided by Javely Biological Products Co., Ltd. (Nanning, China).

2.2. Preparation of HSCCC sample solution

The fruiting body of *A. polytricha* (1.0 kg) was extracted three times with distilled water (15 L) at 90 °C for 3 h. After centrifugation (1700 × *g*, 10 min, 25 °C), the supernatant was concentrated 10-fold, and precipitated with five volumes of absolute ethanol at 4 °C overnight. The precipitate was deproteinated by a combination of enzymolysis with papain and the Sevag method [12], and exhaustively dialyzed against water for 48 h. Then the concentrated dialysate was precipitated with five volumes of absolute ethanol. The precipitate was washed first with absolute ethanol, then acetone, and finally diethyl ether. The solvents were removed from the washed precipitate to yield the crude polysaccharide APPS (75 g). The sample solution for HSCCC was prepared by dissolving 2.0 g of APPS into 50 mL of each phase of a two-phase solvent system

* Corresponding author. Tel.: +86 571 88218710; fax: +86 571 88823509.
E-mail address: qizhendu@163.com (Q. Du).

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composed of PEG1000–K_2HPO_4–KH_2PO_4–H_2O (0.5:1.25:1.25:7.0, w/w).

2.3. Selection of solvent system

The solvent system for HSCCC separation was selected by measurement of partition coefficients of a series of solvent systems composed of different proportions of PEG1000–K_2HPO_4–KH_2PO_4–H_2O. About 1.0 mL of each phase of the PEG1000–K_2HPO_4–KH_2PO_4–H_2O aqueous two-phase system was placed in a test tube, and about 5 mg of the sample was added. The contents were thoroughly mixed and then allowed to settle at room temperature. After two clear layers formed, TLC analysis of the polysaccharide components in the two phases was conducted. The concentration of polysaccharide components in the two phases was estimated by color reaction on TLC, and used to obtain partition coefficients for each polysaccharide component. TLC was performed on a GF 254 plate (Merck, Germany) developed with *n*-butanol–ethanol–water (5:3:2, v/v) and colored with sulfuric acid–ethanol (1:9) at 103 °C. The solvent system that gave partition coefficients of about 0.5–2 for the subject polysaccharide components was selected for HSCCC separation.

2.4. HSCCC separation

A J-type HSCCC instrument made in the Institute of Food and Biological Engineering, Zhejiang Gongshang University, Hangzhou, China, was used to separate the polysaccharide components from *A. polytricha*. The machine holds a separation column at a distance of 10 cm from the center of the centrifuge. The column revolves around the central axis of the centrifuge and simultaneously rotates about its own axis at the same angular velocity in the same direction. The column holder hub was 25 cm in length and 6 cm in O.D. The multilayer coil was prepared by winding 61 m of polytetrafluoroethylene tubing (5.0 mm I.D., Zeus Industrial Products, NJ, United States) onto the holder hub. The β value of the coils from the inner layer to the outer layer was 0.48–0.78. The capacity of the column was determined to be 1200 mL. The mobile phase was delivered using a Waters 510 HPLC pump (Millipore Corporation, Milford, MA, United States). An injection loop (100 mL) was used for sample loading. In each separation, the HSCCC column was first filled with stationary phase, and then the apparatus was started at 700 rpm. The sample of crude extract (2.0 g) dissolved in a mixture of 50 mL each of lower phase and upper phase was injected into the column through an injection loop. Afterward, the mobile phase was delivered into the column at flow rate of 2.5 mL/min to elute the components, and the effluent was collected with a BS-100 mode fraction collector (Shanghai Instrument Factory, Shanghai, China). Each fraction was 15 mL, and was submitted to TLC to analyze the separation. The fractions belonging to the same polysaccharide component were combined and dialyzed with Visking dialysis tubing (molecular weight cutoff 12,000–14,000 Da). After the salt and PEG were removed, the polysaccharide component solution was evaporated under vacuum and freeze-died to obtain the polysaccharide.

2.5. Determination of monosaccharide composition and molecular weight

Gas chromatography (GC) was used for identification and quantification of the monosaccharides. AAPS-2 was hydrolyzed with 2 M TFA at 120 °C for 2 h. The monosaccharides were conventionally converted into the alditol acetates as described previously [13], and analyzed by GC (GC-14A, Shimadzu, Japan) interfaced with a MS (Finnigan Trace MS, Finnigan Co. USA) mass-selective detector at 70 eV ionization energy. The GC column was an OV1701 (30 m × 0.25 mm), with a temperature program from 150 to 250 °C at a rate of 3 °C min^{-1}. The rate of helium carrier gas was 3.0 mL/min. The interface temperature was 250 °C, the ion source temperature was 200 °C and the detective voltage was 350 V. The absolute configurations of the monosaccharides were determined as described by Vliegenthart and co-workers using (+)-2-butanol [14].

The average molecular weight of the polysaccharides was determined by high performance size-exclusion chromatography (HPSEC) [15] on a Waters 2695 pump system fitted with a TSK-G3000PWXL column (7.8 mm I.D. × 30.0 cm) and a Waters 2414 refractive index detector. The data were processed by GPC processing software (Millennium32 version). The mobile phase was 0.7% Na_2SO_4, and the flow rate was 0.5 mL/min at 40 °C, with a pressure of 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 weight was estimated by reference to a calibration curve constructed from a set of Dextran T-series standards of known molecular weight (T-500, 150, 70, 40, 20).

2.6. FTIR analysis

Fourier transform infrared spectroscopy (FTIR) is used to investigate the vibrations of molecules and polar bonds between different atoms. Structures of polysaccharides, including the type of monosaccharide units, glucosidic bonds, and functional groups, can be analyzed using FTIR spectroscopy. Polysaccharide sample (2 mg) was mixed with 250 mg of KBr. The absorption spectra were recorded on a Nicolet Impact 410 Fourier transform infrared spectrophotometer (Thermo Nicolet Co., United States) in the range 400–4000 cm^{-1} at 16 nm/s.

2.7. NMR spectroscopy

For nuclear magnetic resonance (NMR) measurements polysaccharides were dried in a vacuum over P_2O_5 for several days, and then exchanged with deuterium by lyophilizing with D_2O several times [16]. The deuterium-exchanged polysaccharide (15 mg) was put in a 5 mm NMR tube and dissolved in 0.7 mL of 99.96% D_2O. Spectra were recorded with a Bruker Avance 500 spectrometer (Bruker Inc., Switzerland). The ^1H and ^{13}C NMR spectra were recorded at 50 °C. Acetone was used as an internal standard (δ 31.07 ppm) for the ^{13}C spectrum. The ^1H NMR spectrum was recorded by fixing the HOD signal at δ 4.68 ppm at 50 °C.

2.8. Periodate oxidation and Smith degradation [17]

The polysaccharide (10 mg) was dissolved in 0.015 M sodium metaperiodate (25 mL) and kept at 5 °C in the dark for 7 days. The oxidation was stopped by addition of ethylene glycol (1 mL). Consumption of $NaIO_4$ was measured by a spectrophotometric method, and HCOOH production was determined by titration with 0.01 M NaOH. The reaction mixture was dialyzed against distilled water. The retained fraction was reduced by $NaBH_4$ (30 mg, 18 h), neutralized with 0.1 M acetic acid, and dialyzed. The retained fraction from the second dialysis was lyophilized and then hydrolyzed with 2 M trifluoroacetic acid (TFA) at 100 °C for 8 h. The hydrolysate was analyzed by TLC. TLC was performed on a thin layer plate (silica gel G) (Merck, Germany) with a solvent system of *o*-butanol, acetoacetate, iso-propanol, and H_2O (7:4:7:2, v/v). D-Mannose, D-glucose, D-fucose, D-arabinose and D-rhamnose were used as standard sugars. Sugar spots were visualized by spraying with *o*-phthalic acid reagent. Product spots were visualized by spraying with 0.015 M $NaIO_4$, standing for 8 min, and then spraying with benzidine color agent. The benzidine color reagent preparation involved dissolving

0.92 g of benzidine in 50 mL of 50% ethanol and 5 mL of 0.2 M HCl, and then adding 10 mL of acetone.

2.9. Determination of tumor inhibition rate [18]

All animal experiments were carried out in accordance with the guidelines of the institutional ethics committee for animal experiments. Swiss albino female mice 4–6 weeks old and weighing 20–22 g were maintained under standard laboratory conditions. Each mouse was treated orally with polysaccharides (12 mg/kg of body weight) in sterile distilled water (0.5 mL) or only with water (for the model control). Treatment was continued for 6 days/week up to 6 weeks. Tumors were developed in the left hind leg of the mice by intra-muscular injection of 20 μL of Sarcoma-180 cell solution with a concentration of 10^7 cells/mL (2×10^5 cells/mouse). Mice were sacrificed 24 h after the last treatment and the tumors were removed and weighed. Tumor inhibition rate (%) = (tumor weight of the control group − tumor weight of the treatment group)/tumor weight of the control group × 100.

3. Results and discussion

3.1. Solvent system for separation of polysaccharide components

HSCCC is a liquid–liquid partition chromatography where the separation is based on the difference in partition coefficient (K) of solutes within a two-phase solvent system. To achieve efficient separation of polysaccharide components in a high molecular weight complex, it is essential to obtain a large difference of partition coefficient of the isolated components. TLC was used to check the partitioning of the polysaccharide components between the two phases of a series of solvent systems composed of PEG 1000 and potassium phosphate solution in different proportions. $K_{s/m}$ values were calculated by dividing the concentration of each polysaccharide component in upper phase by the concentration in lower phase. The solvent system PEG1000–KH_2PO_4–K_2HPO_4–water (0.5:1.25:1.25:7.0) gave $K_{s/m}$ values of about 0.5, 1 and 1.5 for the three polysaccharide components AAPS-1, AAPS-2 and AAPS-3, respectively. Consequently, this was chosen as the solvent system for separation of the polysaccharide components.

3.2. HSCCC separation

Fig. 1 shows the polysaccharide component analysis of the fractions from HSCCC separation. The sample solution contained 2.0 g of the crude AAPS, and 50 mL of each phase of the two-phase solvent system. The separation was performed at 700 rpm at a flow rate of 2.5 mL/min, using the lower phase as the mobile phase. The solvent front of mobile phase emerged in the 46th tube, which indicates the retention of the stationary phase was 42.5%. Fractions 22–50, 60–84, and 92–110 corresponded to the first (AAPS-1), second (AAPS-2), and third (AAPS-3) polysaccharide components, respectively. The fractions belong to the same polysaccharide component were combined and dialyzed. After the salt and PEG were removed, each polysaccharide component solution was evaporated under vacuum and freeze-died to obtain the three polysaccharides. UV analysis of these three components showed none of them contained proteins or nucleic acids.

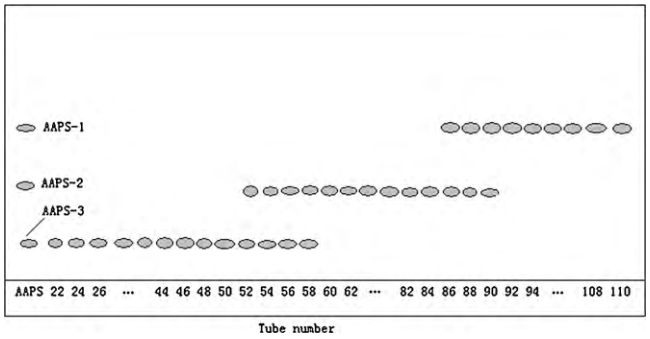

Fig. 1. TLC analysis of fractions (15 mL tubes) from HSCCC separation: fractions 22–50, AAPS-1; fractions 60–84, AAPS-2; and fractions 92–110, AAPS-3. TLC was developed with *n*-butanol–ethanol–water (5:3:2, v/v) and colored with sulfuric acid–ethanol (1:9) at 103 °C.

3.3. Inhibitory effect of polysaccharides on the growth of transplanted tumors in mice

The inhibitory effect of AAPS and its three purified polysaccharides (AAPS-1, AAPS-2, AAPS-3) on the growth of transplanted S180 tumors in mice is shown in Table 1. As a positive control, cyclophosphamide (CTX) showed a high inhibitory rate on S180 sarcoma in mice. The growth of transplanted S180 sarcoma in mice was also significantly inhibited by AAPS. The three purified polysaccharides compared with the model controls ($P < 0.01$) had inhibitory rates of 20.03%, 12.01%, 40.43% and 26.15% for AAPS, AAPS-1, AAPS-2 and AAPS-3, respectively. Among them, AAPS-2 showed the highest inhibitory effect against S180 sarcoma in mice. In addition, no signs of toxicity, which was evaluated on the basis of body weight and microscopic examination of individual organs, were observed in the mice treated with AAPS and its polysaccharides (Table 1).

It is very interesting to obtain some clue to the difference in anticancer activity through an analysis of structure-relationship. However, only the structure of AAPS-2 was successfully elucidated, and no definite structure of AAPS-1 and AAPS-3 was supported by the data of ^{1}H and ^{13}C NMR. In addition, the two polysaccharides showed lower activity in the anticancer test (Table 1). Therefore, the following section mainly introduces the structure elucidation of AAPS-2.

3.4. Structure analysis of AAPS-2

The GC–MS results (Table 2) indicated that the backbone chain was mainly (1 → 3)-linked-β-D-glucosepyranosyl (Residue-A), and (1 → 3, 6)-linked-β-D-glucosepyranosyl (Residue-B). The side chain

Table 1
Inhibitory effect of the crude polysaccharide from *A. polytricha* and its three purified polysaccharides on the growth of transplanted S180 sarcoma in mice.

Group	Dose (mg/kg)	Body weight (g)[a] Before treatment	After treatment	Tumor weight[a]	Inhibitory rate (%)
MC	/	21.0 ± 0.9	22.4 ± 2.8	2.367 ± 0.651	/
AAPS	12	20.5 ± 1.2	22.5 ± 2.4	1.893 ± 0.673[c]	20.0
AAPS-1	12	20.7 ± 0.5	22.6 ± 2.9	2.081 ± 0.576[c]	12.0
AAPS-2	12	21.0 ± 0.9	22.4 ± 2.0	1.410 ± 0.584[c]	40.4
AAPS-3	12	20.5 ± 1.3	22.6 ± 2.8	1.748 ± 0.768[c]	26.2
CTX	12	20.6 ± 0.6	21.9 ± 2.3[b]	1.043 ± 0.627[c]	55.9

Note: a: values are presented as means ± SD ($n = 10$); b: different from the model control (MC), $P < 0.05$; c: different from the model control (MC), $P < 0.01$.

Table 2
Results of methylation analysis of AAPS-2.

Peak No.	Methylated sugar	Molar ratio	Linkage type
1 (Residue-A)	2,4,6-Me_3-Glc*p*	2	→3-β-D-Glc*p*-(1→
2 (Residue-B)	2,4-Me_2-Glc*p*	1	→3,6-β-D-Glc*p*-(1→
3 (Residue-C)	2,3,4,6-Me_4-Glc*p*	1	β-D-Glc*p*-(1→

Table 3
Data from IR and NMR analyses of AAPS-2.

Item	Peaks or signals at
IR analysis	3404.50 cm^{-1}; 2926.05 cm^{-1}; 1635.67 cm^{-1}; 890.27 cm^{-1}
^{1}H NMR analysis	4.46 ppm; 4.15 ppm; 3.98 ppm; 3.64 ppm; 3.42 ppm; 3.25 ppm; 3.21 ppm; 3.20 ppm; 3.05 ppm; 3.01 ppm
^{13}C NMR analysis	103.1 ppm; 86.7 ppm; 86.2 ppm; 76.6 ppm; 76.4 ppm; 75.1 ppm; 73.6 ppm; 72.9 ppm; 70.4 ppm; 69.5 ppm; 68.7 ppm; 61.0 ppm

attached to the O-6 position of Residue-B contained single terminal (1→)-β-D-glucopyranosyl (Residue-C). According to the peak areas, the three types of residues were in a ratio of 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 obtained from periodate oxidation and Smith degradation showed the presence of glucose.

In HPSEC, AAPS-2 showed a single and symmetrically sharp peak (Fig. 2), indicating its homogeneity (data not shown). According to the retention time, its molecular weight was estimated to be 2.59×10^5 Da. It had a negative response to the Bradford test and no absorption at 280 or 260 nm in the UV spectrum obtained on a UV 2550 spectrophotometer (Shimadzu, Japan), indicating the absence of protein and nucleic acid. Total carbohydrate content was determined to be 93%. GC showed AAPS-2 was composed of D-glucose. The FTIR spectral data of AAPS-2 are shown in Table 3. The bands in the region of 3404.50 cm^{-1} were due to the hydroxyl stretching vibration of the polysaccharides. The bands in the region of 2926.05 cm^{-1} were due to the C–H stretching vibration, and the bands in the region of 1635.67 cm^{-1} were due to associated water. The characteristic absorptions at 890.27 cm^{-1} indicated that AAPS-2 contained components in β-configuration (Fig. 3).

In the anomeric region of the ^{1}H NMR spectrum (Table 3) of AAPS-2, two signals occurred at δ 4.46 and δ 4.15 ppm, which were assigned as Residue-A and B, and Residue-C, respectively. Accordingly, in the anomeric region of the ^{13}C NMR spectrum (Table 3), four carbon resonances appeared at δ 103.1 ppm. All the results confirmed the presence of the three sugar residues (A, B, and C) and their configurations (β), which are consistent with the GC and FTIR data. In the high magnetic field, the δ 86.7, δ 86.2 and δ 76.6 ppm

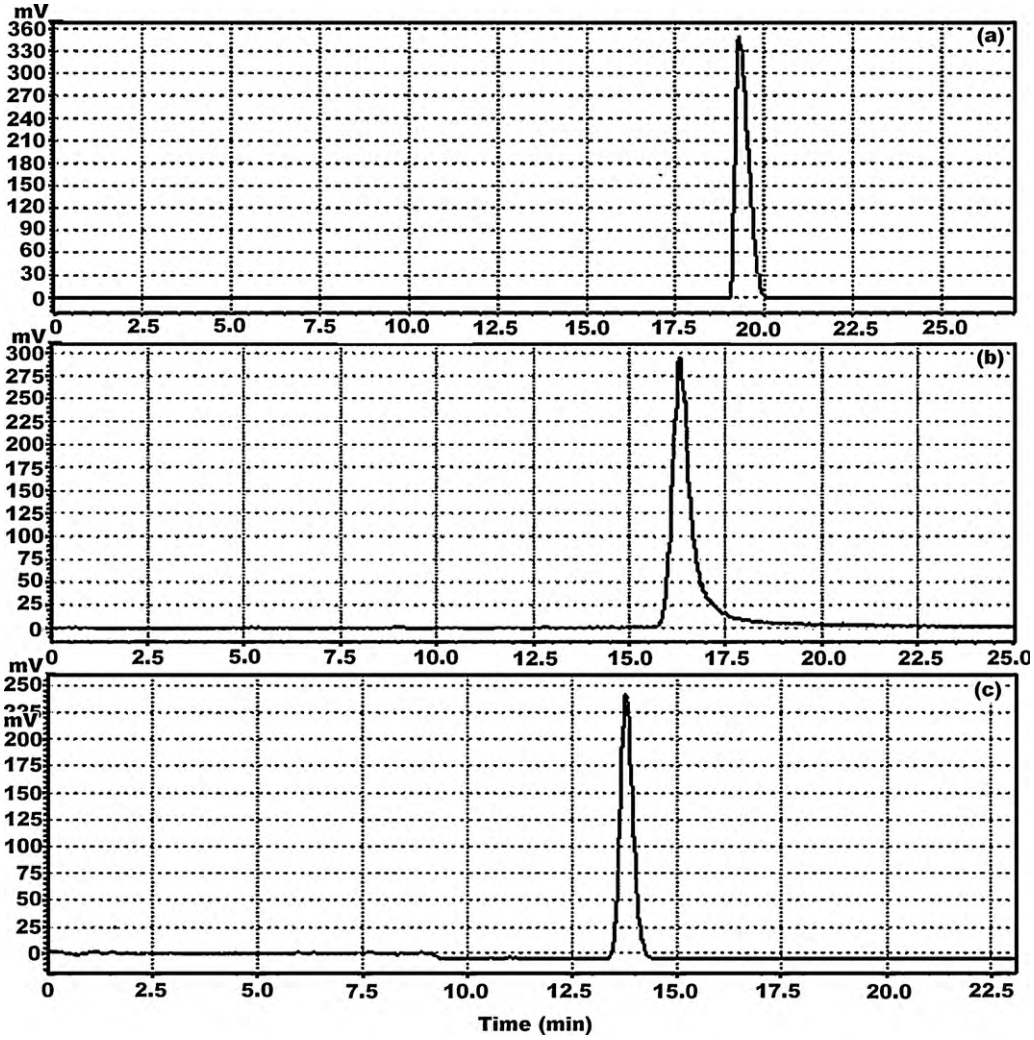

Fig. 2. GPC-HPLC analysis of AAPS-1 (a), AAPS-2 (b), and AAPS-3 (c) from *A. polytricha*. Chromatography conditions were as follows: column, Waters HPLC Ultrahydrogel Linear column (7.8 × 300 mm); column temperature, 30 °C; mobile phase, pure water; flow rate, 0.5 mL/min; detection conditions; refractive index detector; detection temperature, 40 °C.

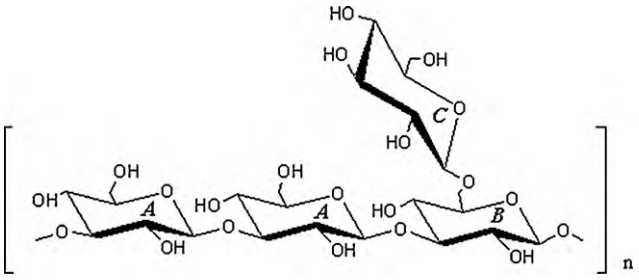

Fig. 3. Structure of AAPS-2.

signals should come from the C-3 resonance of residues A, B and C, respectively. C-6 chemical shifts of Residue-A, Residue-B and Residue-C occurred at δ 61.0, δ 69.5 and δ 61.0 ppm, respectively. All the NMR chemical shifts were compared with literature values [19–22]. The structure of this polysaccharide was elucidated as follows: the backbone was composed of (1 → 3)-linked-β-D-glucopyranosyl and (1 → 3, 6)-linked-β-D-glucopyranosyl residues in a ratio of 2:1, and terminated with one (1→)-β-D-glucopyranosyl at the O-6 position of (1 → 3, 6)-linked-β-D-glucopyranosyl along the main chain (Fig. 3).

4. Conclusion

Three polysaccharides (AAPS-1, AAPS-2 and AAPS-3) were separated from a crude extract of *A. polytricha* by HSCCC. Separation was achieved using a two-phase aqueous solvent system composed of PEG and a saline solution. AAPS-2 showed excellent inhibition against growth of S180 sarcoma in mice. This study demonstrates that HSCCC is an effective method for preparation of bioactive polysaccharides.

Acknowledgments

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References

[1] Y.X. Sun, J.C. Liu, Int. J. Biol. Macromol. 43 (2008) 279.
[2] Y. Murata, T. Shimamura, T. Tagami, F. Takatsuki, J. Hamuro, Int. Immunopharmacol. 2 (2002) 673.
[3] N. Markova, V. Kussovski, I. Drandarska, S. Nikolaeva, N. Georgieva, T. Radoucheva, Int. Immunopharmacol. 3 (2003) 1557.
[4] X. Luo, M.Y. Yu, N. Jiang, X.Y. Xu, J. Zeng, L.Y. Zheng, Jun Wu Xi Tong 28 (2009) 435.
[5] J.L. Mau, G.R. Chao, K.T. Wu, J. Agric. Food Chem. 49 (2001) 5461.
[6] B.K. Yang, J.Y. Ha, S.C. Jeong, Y.J. Jeon, K.S. Ra, S. Das, Biotechnol. Lett. 24 (2002) 1319;
M. Tani, H. Tanimura, H. Yamaue, T. Tsunoda, M. Iwahashi, K. Noguchi, Anticancer Res. 13 (1993) 1773.
[7] T. Taguchi, Cancer Detect. Prev. Suppl. 1 (1987) 333.
[8] H. Nakano, K. Namatame, H. Nemoto, H. Motohashi, K. Nishiyama, K. Kumada, Hepatogastroenterology 46 (1999) 2662.
[9] M. Suzuki, T. Kikuchi, F. Takatsuki, J. Hamuro, Cancer Immunol. Immunother. 38 (1994) 1.
[10] Y. Ito, in: Y. Ito, W.D. Conway (Eds.), High-Speed Countercurrent Chromatography, Wiley-Interscience, New York, 1996.
[11] Y. Shibusawa, S. Iino, H. Shindo, Y. Ito, J. Liq. Chromatogr. Rel. Technol. 24 (2001) 2007.
[12] Y.X. Sun, S.S. Wang, T.B. Li, X. Li, L.L. Jiao, L.P. Zhang, Biores. Technol. 99 (2008) 900.
[13] T.M. Johnes, P. Albersheim, Plant Physiol. 49 (1972) 926.
[14] G.J. Gerwig, J.P. Kamerling, J.F.G. Vliegenthart, Carbohydr. Res. 77 (1979) 1.
[15] Y.X. Sun, H.T. Liang, X.T. Zhang, H.B. Tong, J.C. Liu, Biores. Technol. 100 (2009) 1860.
[16] M.T. Dueñas-Chasco, M.A. Rodríguez-Carvajal, P.T. Mateo, G. Franco-Rodríguez, J. Espartero, A. Irastorza-Iribas, Carbohydr. Res. 303 (1997) 453.
[17] A. Linker, L.R. Evans, G. Impallomeni, Carbohydr. Res. 335 (2001) 45.
[18] C. Liu, Q. Lin, Y. Gao, L. Ye, Y. Xing, T. Xi, Carbohydr. Polym. 67 (2007) 313.
[19] E.R. Carbonero, A.H.P. Gracher, F.R. Smiderle, F.R. Rosado, G.L. Sassaki, P.A.J. Gorin, M. Iacomini, Carbohydr. Polym. 66 (2006) 252.
[20] J.C. Santos-Neves, M.I. Pereira, E.R. Carbonero, A.H.P. Gracher, P.A.J. Gorin, G.L. Sassaki, M. Iacomini, Carbohydr. Res. 343 (2008) 1456.
[21] T. Ali, A. Weintraub, G. Widmalm, Carbohydr. Res. 341 (2006) 1878.
[22] S. Mondal, I. Chakraborty, D. Rout, S.S. Islam, Carbohydr. Res. 341 (2006) 878.