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Structural analysis of an alkali-extractable and water-soluble polysaccharide (ABP-AW1) from the fruiting bodies of *Agaricus blazei* Murill

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ABSTRACT

An alkali-extractable and water-soluble polysaccharide (ABP-AW1) was isolated by 5% sodium hydroxide solution from the fruiting bodies of *Agaricus blazei*. ABP-AW1 (M_w = 50 kDa), was composed of Gal, Glc, Fuc, Ara and Man with the molar ratio of 29: 20: 6: 2: 2. According to the combination of chemical and instrumental analysis, the results indicated that ABP-AW1 had a backbone consisting of (1 → 6)-linked-β-D-galactopyranosyl, (1 → 6)-linked-β-D-glucopyranosyl and (1 → 3, 6)-linked-β-D-glucopyranosyl, which was terminated with (1→)-linked Fuc, Ara and Man residues at the O-3 position of (1 → 3, 6)-linked-β-D-glucopyranosyl in the proportion of 29: 10: 10: 6: 2:2.

1. Introduction

The Basidiomycete fungus *Agaricus blazei*Murill has originally used as a folk remedy and food in Brazil. It was also known for its potent anti-tumor activity (Takaku, Kimura, & Okuda, 2001; Ziliotto, Pinheiro, Barbisan, & Rodrigues, 2009). Many studies had proved that its glycoprotein was to be as an antitumor-active principle, which showed strong effects on treating and preventing cancer (Dong, Yao, Yang, & Fang, 2002; Liu et al., 2010). Previously one remarkable antitumor components, FIII-2-b, was prepared from the sodium hydroxide extract of the fruiting bodies of *A. blazei*. The structure was analyzed to be (1 → 6)-β-D-glucan-protein complex by methylation and NMR analyses (Kawagishi et al., 1989). From the extraction scheme, FIII-2-b is a part of an alkali-extractable and water-soluble glycoprotein, which was directly one-step purified by 0.3 M sodium hydroxide solution by Toyopearl HW55S gel filtration chromatography. However, in our present research we designed a more detailed process route to purify the alkali-extractable and water-soluble polysaccharides than ever. Taking advantage of size-exclusion chromatography and ion-exchange chromatography on an ÄKTA explore 100 purification system, we obtained one neutral polysaccharide unlike others. Therefore we initiated this research on the structural elucidation of this new polysaccharide.

2. Experimental

2.1. Materials

The fruiting bodies of *A. blazei* were purchased from Zhengjiang Bo Rui Pharmaceuticals Co., Ltd. (Zhejiang, China). DEAE Sepharose Fast Flow, Sepharose 6 Fast Flow, and Sephadex G-25 were purchased from Amersham (Sweden). D-glucose was form Amresco Inc. T-series dextran was purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were of analytical grade.

2.2. General methods

UV–vis absorption spectra were recorded with a UV–vis spectrophotometer (Model SP-752, China). GC was performed on a Shimadzu GC-14C instrument (Shimadzu, Japan) equipped with a 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 a DM-2330 capillary column (30 m × 0.32 mm × 0.20 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 the phenol–H_2SO_4 method, with glucose as the standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Protein was measured by the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951), with bovine serum albumin (BSA) as the standard. Dialysis was carried out using tubing with a M_w cut-off of 500 Da (for globular proteins).

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2.3. Isolation and purification of polysaccharide

The fruiting bodies of *A. blazei* were extracted with 95% ethanol (5000 ml, ×3) at 75 °C for 6 h under reflux to remove lipid. The residue was then extracted with distilled water (8000 ml) at 75 °C for 3 times and 3 h for each time. Then the water unextractable solid was washed, dried and extracted with 0.5 M NaOH solution which contained 0.3% (w/w) $NaBH_4$ at room temperature for overnight two times, and the extraction solution was filtered through line cloth. The suspension was neutralized with hydrochloric acid (0.1 M) and filtered. The supernatant containing water-soluble polysaccharide was dialyzed, concentrated, ethanol precipitation and then dried. The precipitate collected by centrifugation (4000rpm; 10 min) was deproteinated by proteinase digestion and the Sevag method (Sun et al., 2008), followed by exhaustive dialysis with water for 48 h. Then the concentrated dialyzate was precipitated with 4 vol of 95% EtOH at 4 °C for 24 h. The precipitate was washed with absolute ethanol, acetone, and ether. The washed precipitate was the crude polysaccharide (CABP-AW).

The CABP-AW was purified on an ÄKTA explore 100 purification system equipped with a pump P-900, a UV-900 monitor, a pH/C-900 monitor, a Fraction Collector Frac-950, and an autosampler A-900. The CABP-AW was dissolved in distilled water, centrifuged (4000 rpm; 10 min), and then the supernatant was applied to a DEAE Sepharose Fast Flow column (2.6 × 40 cm) equilibrated with ultrapure water. After loading with sample, the column was eluted with different stepwise gradient of NaCl aqueous solutions (0, 0.2, 0.4, and 0.6 M) at a flow rate of 4 ml/min, respectively. Different fractions (8 ml in each tube) were collected using the Frac-950, and then purified further on a Sepharose 6 Fast Flow column (2.6 × 100 cm) with 0.15 M/L NaCl at a flow rate of 1 ml/min to yield three main fractions (ABP-AWs), coded as ABP-AW1, ABP-AWA1 and ABP-AWB1, and then were applied to a Sephadex G-25 column (2.6 × 40 cm) to remove salts. All the fractions were collected, dialyzed and lyophilized to give white purified polysaccharide fractions. Total carbohydrate content of each tube was measured at 490 nm by Dubois's method, and protein absorption at 280 nm was measured for each fraction.

2.4. Partial hydrolysis with acid

The ABP-AW1 (100 mg) was hydrolyzed with 0.05 M TFA (3 ml) at 95 °C for 10 h, and then centrifuged. Afterward, TFA was removed by evaporation, and the rest was dialyzed with distilled water for 48 h, and then the solution was diluted in the sack with ethanol. After hydrolysis, the precipitate and supernatant in the sack and the fraction out of sack were dried and analyzed by GC, as was done with the alditol acetate. The precipitate in the sack was subjected to monosaccharide composition and methylation analyses (Sun, Liu, Yang, & Kennedy, 2010).

2.5. Periodate oxidation and Smith degradation

For analytical purposes, 25 mg of the polysaccharide was dissolved in 12.5 ml of distilled water, and 12.5 ml of 30 mM $NaIO_4$ were added. The solution was kept at 4 °C for 7days 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 spectrophotometer 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 10 ml volume. One-third of this solution was freeze-dried and analyzed with GC. The rest was 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 before, and the content outside the sack was desiccated for GC analysis; the content inside was diluted with ethanol, and after centrifugation, the supernatant and precipitate were also dried for the GC analysis.

2.6. Monosaccharide composition, homogeneity and M_w determination of polysaccharides

Gas chromatography (GC) was used for identification and quantification of the monosaccharides. ABP-AW1 was hydrolyzed with 2 M trifluoroacetic acid (TFA) at 120 °C for 2 h. The monosaccharides were conventionally converted into the alditol acetates as described (Jones & Albersheim, 1972; Oades, 1967) and analyzed by GC as previously mentioned.

The average molecular weight of ABP-AW1 was determined by high-performance size-exclusion chromatography (HPSEC) (Sun & Liu, 2009), which was performed on a SHIMADZU HPLC system fitted with one TSK-G3000PW_{XL} column (7.8 mm ID × 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 at 40 °C. 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 were injected. The molecular mass was estimated by reference to a calibration curve made from a set of Dextran T-series standards of known molecular masses (T-130 80, 50, 25, 10).

2.7. Methylation analysis

The sample (20 mg) was methylated three times, according to 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, then reduced and acetylated as described by Sweet, Shapiro, & Albersheim (1975). The partially methylated alditol acetates were analyzed by GC–MS under the same chromatographic conditions described above.

3. Results and discussion

3.1. Isolation, purification and chemicophysical properties of ABP-AW1

ÄKTA explore 100 purification system was successfully employed to purify the alkali-soluble and water-soluble polysaccharide (ABP-AW1) from the fruiting bodies of *A. blazei*. According to charge differences, we designed five different stepwise gradients of NaCl aqueous solutions (0, 0.2, 0.4 and 0.6 M) to elute the alkali-extractable and water-soluble fractions through the DEAE Sepharose Fast Flow column yielding one neutral and two weak anion peaks. And then based on molecular weight difference, the neutral part eluted by water solution was purified further on a Sepharose 6 Fast Flow column (2.6 × 100 cm) with 0.15 M NaCl at a flow rate of 1.5 ml/min yielding one fraction (ABP-AW1), and then were applied to a Sephadex G-25 column (2.6 × 40 cm) to remove salts. The ABP-AW1 showed a single and symmetrically sharp peak, indicating its homogeneity on HPSEC (Fig. 1). According to the retention time, its molecular weight was estimated to be 50 kDa.

Total carbohydrate content was determined to be 93.4%. 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. The ABP-AW1 was composed of Gal; Glc;

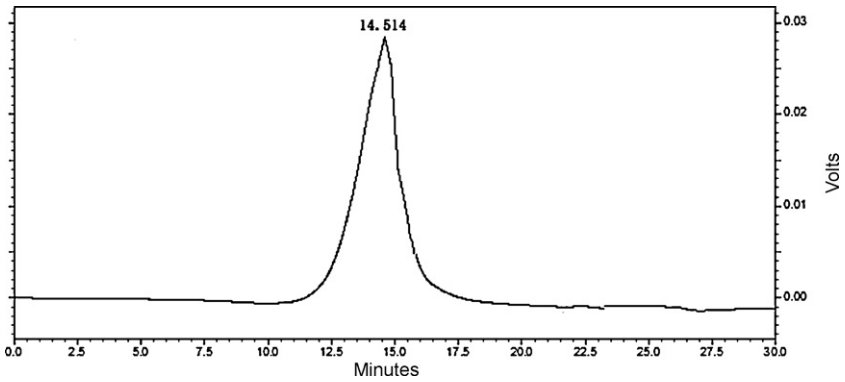

Fig. 1. The average molecular weight and homogeneity of ABP-AW1 was determined by high-performance size-exclusion chromatography (HPSEC), eluting with 0.7% Na_2SO_4 at a flow rate of 0.5 mL/min.

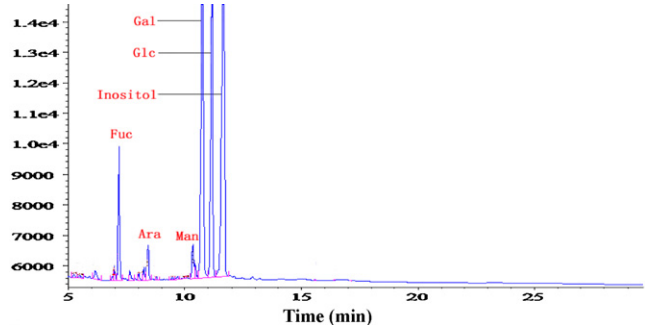

Fig. 2. GC profile of ABP-AW1. Peaks from left to right: Fuc, Ara, Man, Gal, Glc, Inositol.

Fuc; Ara; Man, as detected by GC in the molar ratio of 29: 20: 6: 2: 2 (Fig. 2).

3.2. Structural analysis of ABP-AW1

The data on IR are showed in Fig. 3. The bands in the region of 3423.42 cm^{-1} are due to the hydroxyl stretching vibration of the polysaccharides. The bands in the region of 2914.83 cm^{-1} are due to C–H stretching vibration, and the bands in the region of 1631.61 cm^{-1} are due to associated water. Moreover, the characteristic absorptions at 889.36 cm^{-1} in the IR spectra indicated β-configurations existing in ABP-AW1.

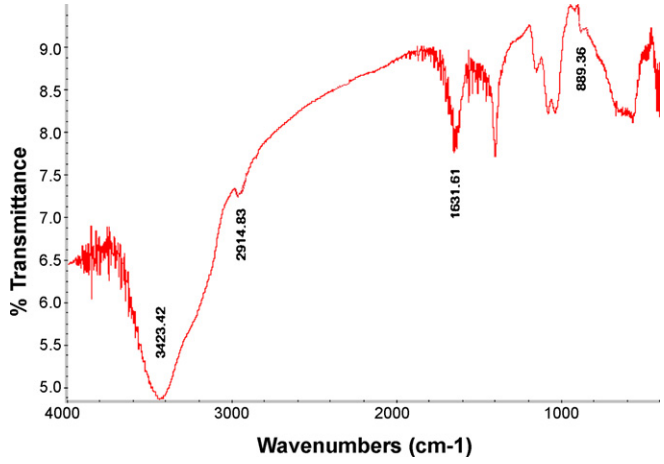

Fig. 3. The FTIR spectra of ABP-AW1 isolated from the fruiting bodies of *A. blazei* were recorded on SPECORD in a range of 400–4000 cm^{-1}.

Table 1
The results of methylation analysis of ABP-AW1.

Peak no.	Methylated sugar	Molar ratio	Linkage type
1 (Residue-A)	2,3,4-Me_3-Gal	20	→6)-β-D-Gal*p*-(1→
2 (Residue-B)	2,3,4-Me_3-Glc	10	→6)-β-D-Glc*p*-(1→
3 (Residue-C)	2,4-Me_2-Glc	10	→3, 6)-β-D-Glc*p*-(1→
4 (Residue-D)	2,3,4,6-Me_4-Fuc	6	β-L-Fuc*p*-(1→
5 (Residue-E)	2,3,4,6-Me_4-Ara	2	β-L-Ara*f*-(1→
5 (Residue-F)	2,3,4,6-Me_4-Man	2	β-D-Glc*p*-(1→

The GC–MS results (Table 1) indicated that the backbone chains are mainly (1 → 6)-linked-β-D-galactopyranosyl (Residue-A), (1 → 6)-linked-β-D-glucopyranosyl (Residue-B) and (1 → 3, 6)-linked-β-D-glucopyranosyl (Residue-C). The side chains attached to the O-3 position of Residue-C contained three terminal units, namely (1→)-β-L-fucopyranosyl (Residue-D), (1→)-β-L-arabinofuranose (Residue-E) and (1→)-β-D-glucopyranosyl (Residue-F). According to the peak areas, six types of residues are in the ratio of 20: 10: 10: 6: 2: 2. 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–Smith degradation showed the presence of glycerol, in addition to plenty of formic acid.

4. Conclusion

In conclusion, the structural feature of ABP-AW1 from the fruiting bodies of *A. blazei* was successfully characterized by means of chemical analyses and instrumental spectroscopy. The results demonstrated that the backbone of ABP-AW1 consisted of (1 → 6)-linked-β-D-galactopyranosyl, (1 → 6)-linked-β-D-glucopyranosyl and (1 → 3,6)-linked-β-D-glucopyranosyl residues, and branched with three terminal (1→)-β-L-fucopyranosyl, (1→)-β-L-arabinofuranose and (1→)-β-D-glucopyranosyl units at the O-3 position of (1 → 3,6)-linked-α-D-glucopyranosyl along the main chain in the ratio of 20: 10: 10: 6: 2: 2. The further detailed structure elucidation would continue in our later research.

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