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Research article

Isolation, purification, and structural elucidation of a water-soluble polysaccharide derived from *Phellinus baumii* Pilát mycelia

JianGuang Luo [a,b], JunYan Wu [a], YiNi Xu [a], Hu Shao [a,**,1], Ping-Hsiu Huang [a,*,1]

[a] *School of Food, Jiangsu Food and Pharmaceutical Science College, No.4, Meicheng Road, Higher Education Park, Huai'an City, Jiangsu Province, 223003, China*

[b] *Jiangsu Engineering Research & Development Center for Food Processing, Huai'an, Jiangsu, 223003, China*

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ABSTRACT

This study involved the successful production of mycelial polysaccharides by optimizing submerged culture conditions of *Phellinus baumii* Pilát. Then, the investigation focused on the composition and chemical structure of the water-soluble polysaccharide from *P. baumii* Pilát mycelia (PBMP) by extraction and purification. Specifically, this study indicated that a water-soluble PBMP fraction 1 ($PBMP_1$) was isolated from PBMP. Moreover, this study discovered a $PBMP_1$ isolated from PBMP, which was found to have the monosaccharide compositions comprised of fucose (Fuc), glucose (Glc), and galactose (Gal) despite the absence of proteins. Subsequently, the composition and structure of $PBMP_1$ were characterized using Fourier transform infrared (FT-IR) spectroscopy, periodate oxidation, Smith degradation, methylation reaction, gas chromatography-mass spectrometer (GC-MS), and Nuclear magnetic resonance (NMR). The composition and structure of $PBMP_1$ were characterized in this study, revealing an α-glycosidic bond conformational linkage with 1,4-Glc residues and 1,6-Gal residues forming the backbone. Additionally, a highly branched hetero-polysaccharide was identified with a non-reducing terminus of Fuc containing 1,3,4- and 1,4,6-Glc branching. The findings of this study offer valuable insights and information that can be utilized by researchers, manufacturers, and other stakeholders to advance the field of *P. baumii* Pilát product development. Moreover, these results have significant implications for future large-scale mass production and functional applications of $PBMP_1$ products.

1. Introduction

P. baumii Pilát, a basidiomycete fungus belonging to the family of *Hymenochaetaceae*, is a precious and highly acclaimed medicinal fungus [1]. It has been reported that polysaccharides isolated from the fruiting body (also called SangHuang) of *P. baumii* Pilát possess a range of biological activities, including antioxidant, anti-obesity, anti-inflammatory, antiplatelet, immune-stimulating, and

* Corresponding author.
** Corresponding author.
E-mail addresses: 20101003@jsfpc.edu.cn (J. Luo), 20061016@jsfpc.edu.cn (J. Wu), 20051007@jsfpc.edu.cn (Y. Xu), shzj0201@163.com (H. Shao), hugh0530@gmail.com (P.-H. Huang).
[1] These authors contributed equally to this work.

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Abbreviations

Polysaccharide from *P. baumii* Pilát mycelia (PBMP)
PBMP fraction 1 ($PBMP_1$)
Fucose (Fuc)
Glucose (Glc)
Galactose (Gal)
Fourier transform infrared (FT-IR)
Gas chromatography-mass spectrometer (GC-MS)
Nuclear magnetic resonance (NMR)
Molecular weight (MW)
Mannose (Man)
China Forestry Culture Collection Center (CFCC)
High-performance gel permeation chromatography (HPGPC)
High-performance liquid chromatography (HPLC)
Refractive index detector (RID)
Trifluoroacetic acid (TFA)
Flame ionization detector (FID)
Potassium bromide (KBr)
Electron ionization (EI)
Chemical shift (δ)
Splitting constant (J)
Standard deviation (SD)
International Business Machines Corporation (IBM)
Analysis of Variance (ANOVA)
Fraction (F)
Retention time (RT)
Sustainable Development Goals (SDGs)

antitumor properties [2–4]. In addition, the anti-diabetic effect and immune-stimulating activity of exo-polysaccharides from the submerged mycelial culture of *P. baumii* Pilát have also been reported [5,6]. Remarkably, it has been reported that the polysaccharides in *P. baumii* Pilát intimately associated with lignans would be complex to extract by typical approaches [7]. The same authors have reported extracting and purifying a hetero-polysaccharide as *P. baumii* Pilát-2 (with the most significant percentage of Glc) by acid-chlorite delignification. Regarding polysaccharide extraction, it has been reported that different cultivation methods result in substantial differences in monosaccharide compositions and molar ratios [8–10]. Simultaneously, the fractions precipitated by ethanol exhibited complexity in the monosaccharide compositions [9,11,12]. In addition, the biological activity of PBMP has also been reported to be markedly affected by the chemical structure of PBMP, specifically molecular weight (MW), uronic acid content, solubility, spatial configuration, polymer charge, sulfate content, types of glycosidic bonds and branches, etc., [13,14]. Thus, novel and potentially exploitable hetero-polysaccharides can be identified for any PBMP obtained from different incubations and utilized to purify further.

Despite previous studies revealing that these bioactive potencies result from the contribution of polysaccharides, polyphenols, sesquiterpene, and undescribed alkyl-benzene derivatives [1,3,4,9]. Notably, these bioactives have demonstrated diverse health-promoting bioactivities encompassing antioxidant potential, anti-inflammatory properties, regulation of gut microbiota, and beneficial effects against cancer [15,16]. Owing to its perceived health benefits, *P. baumii* Pilát has long been utilized as a traditional herbal medicine in East Asian countries. However, this team also reported the culture conditions, preliminary characterization, and antioxidant activity *in vivo* of crude PBMP [6]. The published results in previous studies by this team have reported that the crude PBMP had potent antioxidant activity [5,6]. The specific purification and structure information on the PBMP is still poorly understood [14]. To maximize the utilization of this novel resource, thoroughly examining the structural characteristics of crude PBMP derived from *P. baumii* Pilát is imperative. Therefore, the objectives of this study were to investigate the isolation, purification, and structural properties of this water-soluble PBMP. This study utilized the previously developed *P. baumii* Pilát-optimized culture conditions [5,6] for mass preparation of PBMP as raw material for $PBMP_1$ extraction and purification, which performs the composition's physico-chemical analysis.

2. Materials and methods

2.1. Chemical reagents and strain

Fuc, mannose (Man), Glc, Gal, inositol, DEAE-cellulose 52, Sephadex G-100, and other chemical reagents were purchased from Sigma-Aldrich® (Merck KGaA, Darmstadt, Germany). The strain of *P. baumii* Pilát was purchased from the China Forestry Culture

Collection Center (CFCC, Beijing, China).

2.2. Preparation of mycelial polysaccharide

2.2.1. Culture of P. baumii Pilát

P. baumii Pilát was maintained and cultured as described by Luo et al. [5]. The seed culture was grown in a 250 mL Erlenmeyer flask containing 50 mL of basal medium (Glc 20 g/L, peptone 2 g/L, yeast extract 1 g/L, KH_2PO_4 1 g/L, $MgSO_4$ 0.5 g/L, thiamine 0.01 g/L, distilled water, initial pH 6.0) at 28 °C on a rotary incubator at 150 rpm for 7 days. The submerged cultures were performed in 500 mL Erlenmeyer flasks containing 100 mL of medium (Glc 35.36 g/L, yeast extract 2.88 g/L, peptone 2.73 g/L, $MgSO_4$ 1.47 g/L, KH_2PO_4 1 g/L, VB_1 0.0075 g/L and diammonium oxalate monohydrate 0.3 g/L) after inoculating with 10 % (*v/v*) of the seed culture at 28 °C on a rotary shaker at 150 rpm for 6 days.

2.2.2. Extraction and purification of $PBMP_1$

PBMPs were isolated according to this team's previous reports [6]. The PBMP was harvested by filtering through a filter paper to separate it from the liquid medium. Afterward, the mycelial pellets were washed with deionized water, repeated thrice, followed by drying in an oven at 60 °C until constant weight was reached. The dried mycelia were ground into a fine powder with an electric mill and extracted thrice for 2 h each with distilled water at 90 °C (the ratio of dry mycelia and distilled water was 1:20; *w/v*). The water-insoluble material was filtered and centrifuged. The supernatants were collected and concentrated into one-fifth of the original volume. The concentrated liquors were mixed with three times the volume of absolute ethanol, stirred vigorously, and kept at 4 °C overnight. The precipitate was collected by centrifugation, washed twice with anhydrous ethanol, acetone, and ether, and dried to obtain a crude PBMP.

The crude PBMP (100 mg) was redissolved in 5 mL distilled water (*w/v*), applied to DEAE-cellulose 52 column (2.6 × 30 cm), eluted with deionized Water, and then 0.1 and 0.3 M NaCl, respectively. Based on the colorimetric test performed for total carbohydrates by the phenol-sulfuric acid method [17], the fraction eluted with distilled water was further purified on a Sephadex G-100 column (2.6 × 60 cm) eluted with 0.1 M NaCl at a 0.25 mL/min flow rate. The main fraction was collected, dialyzed, and then lyophilized to get a white-purified mycelial polysaccharide of $PBMP_1$.

2.3. Physicochemical properties of $PBMP_1$

2.3.1. Determination of total carbohydrate content, uronic acid, and protein contents

Protein content was analyzed by the method of Bradford [18], and total carbohydrate content was performed by phenol-sulfuric acid method [17] using bovine serum albumin and D-Glc as standard, respectively. Uronic acid content was determined by the *m*-hydroxyphenyl colorimetric procedure with D-glucuronic acid as the standard [19].

2.3.2. Determination of homogeneity and molecular weight

The homogeneity and MW of $PBMP_1$ were determined by the high-performance gel permeation chromatography (HPGPC) method on a high-performance liquid chromatography (HPLC) system (1100, Agilent Technologies, Santa Clara, CA, USA) equipped with a TSK-GEL column (G3000-SWxl, 7.5 × 300 mm, Tosoh Co., Tokyo, Japan) and a refractive index detector (RID). The sample solution (20 μL, 0.2 %, *w/v*) was injected in each run, with 0.1 M Na_2SO_4 solution as the mobile phase at a flow rate of 0.8 mL/min. The standard curve was prepared with pullulan standards of MW range at 0.59–78.8 × 10^4 Da (Shodex standard P-82, Resonac Holdings Co., Tokyo, Japan) and repeated as described above, then by interpolating to calculate the monosaccharides content in $PBMP_1$.

2.3.3. Monosaccharide composition analysis

GC was used to identify and quantify the monosaccharides. The $PBMP_1$ (5 mg) was hydrolyzed with 2 mL of 2 M trifluoroacetic acid (TFA) at 120 °C for 2 h. Afterward, the hydrolyzate was repeatedly co-distillated with methanol to a certain degree of dryness and conventionally converted into the aldonitrile acetates based on previous descriptions [6]. The monosaccharides standards were also operated as above and analyzed on a GC equipped (6890N, Agilent Technologies) with a flame ionization detector (FID) and an HP-5 fused silica capillary column (30 m × 0.32 × 0.25 mm). The specific conditions were as follows: nitrogen gas was used as the carrier gas at a flow rate of 1 mL/min; the injector and detector temperature settings were set to 250 and 280 °C, respectively; the injection volume was 1.0 μL; the column temperature was maintained at 120 °C for 2 min, subsequently programmed at 3 °C/min to 210 °C and maintained for 4 min. Monosaccharide standards (Fuc, Glc, and Gal) were used to prepare standard curves by repeating all the above operations and calculating the individual monosaccharide contents in $PBMP_1$ by interpolation.

2.4. Structural characterization of $PBMP_1$

2.4.1. FT-IR spectrometric analysis

Determining the functional groups of the $PBMP_1$ was performed based on the approach described in Huang et al. [20]. The 1 mg $PBMP_1$ was mixed with the dried potassium bromide (KBr) powder and then obtained by pressing it in a mold. In addition, the background was scanned using KBr (without $PBMP_1$). Subsequently, the IR spectra (4000–400 cm^{-1}, frequency of 64 scans) were analyzed using an FT-IR spectrometer (Nicolet 6700, Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.4.2. Periodate oxidation-Smith degradation reaction

Periodate oxidation and Smith degradation were performed according to the previously described method [21,22]. The 25 mg of $PBMP_1$ was dissolved in 25 mL of 15 mM $NaIO_4$, and the mixture was evenly mixed and then transferred to 4 °C (hourly with intermittent oscillations). The whole process required to be carried out away from light. The sample was collected at 6 h intervals by sampling 0.1 mL and diluted to 25 mL, where the absorbance at 223 nm of the sample was determined using a spectrophotometer (UV-2450, SHIMADZU Co., Kyoto, Japan). The complete oxidation was identified with a stable absorbance within 96 h, while the spectrophotometry measurement was conducted to determine the consumption of $NaIO_4$. Ethylene glycol (2 mL) was incorporated to conclude the periodate oxidation reaction. In addition, the level of formic acid was evaluated by titration with 0.5 mM sodium hydroxide using a portion of the periodate-oxidized product (2 mL). Subsequently, the remaining periodate product was dialyzed using tap water (48 h) followed by distilled water (24 h). The non-dialysates were concentrated for reduction with sodium borohydride (50 mg) for 24 h at room temperature, followed by adjusting the pH to 6.0 via the 0.1 M acetic acid and dialyzed as described above. Concisely, the obtained product was hydrolyzed with 4 mL of 2 M TFA at 120 °C for 2 h and analyzed by GC under the same conditions as above (Section 2.3.3).

2.4.3. Methylation analysis

Methylation analysis of the sample was performed according to Ciucanu and Kerek's [23] description. Triple methylation was applied to $PBMP_1$ (10 mg) and confirmed by the disappearance of the hydroxyl peak around 3400 cm^{-1} in the FT-IR (Nicolet 6700, Thermo Fisher Scientific Inc.) spectrum. Next, the permethylated polysaccharide was hydrolyzed using 2 M TFA at 120 °C for 2 h, while the resulting hydrolysates were reduced by using $NaBH_4$ and acetylated with acetic anhydride. Next, analyze the above samples using Varian CP-3800 GC with Saturn 2200 GC/ion trap MS (Varian Medical Systems, Inc. Palo Alto, CA, USA) equipped with a DB-5 fused silica capillary column (30 m × 0.25 mm × 0.25 μm). Specific conditions were analyzed: The temperature program was the same as for the GC. The relative retention time and fragmentation pattern were used to identify linkages. The mass spectrometer was operated with an electron ionization (EI) source set at 70 eV, a multiplier voltage of 350 V, a filament current of 250 μA, an interface temperature of 260 °C, and an ion source temperature of 180 °C. The mass-to-charge ratio (m/z) scanning range was between 30 and 450, with a scan rate of 2.5 scan/sec.

2.4.4. NMR analysis

$PBMP_1$ was deuterium exchanged three times by freeze-drying from D_2O and then dissolved in 99.96 % D_2O at concentrations of 5 mg/mL for ^1H NMR and 20 mg/mL for ^{13}C NMR. The spectra were performed using the NMR spectrometers (DRX-500 and AV-300, Bruker Co., Billerica, MA, USA). The chemical shift (δ) was expressed in 10^{-6}, and the splitting constant (J) was expressed in Hz. The δ at ^1H refers to residual D_2O at 4.75 ppm (25 °C) as an internal standard, while for ^{13}C, it relates to an externally calibrated DSS (d 0.00 ppm).

2.5. Statistical analysis

Unless otherwise indicated, all results in this study were presented as mean ± standard deviation (SD). The data statistical analysis in this study was performed using the packaged software Statistical Product and Service Solutions (SPSS; V12, International Business Machines Corporation (IBM) Armonk, New York, USA). The data were subjected to statistical analyzed using the one-way Analysis of Variance (ANOVA). *P*-values below 0.05 were regarded as statistically significant.

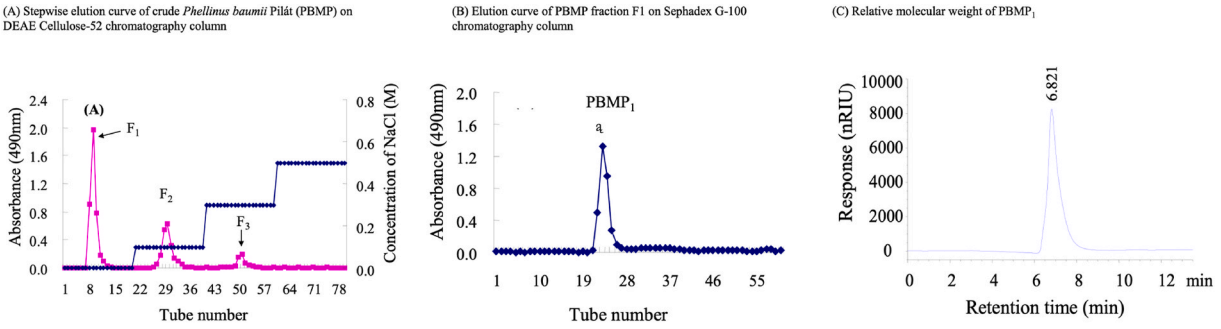

Fig. 1. Isolation, purification, and composition of polysaccharide from *Phellinus baumii* Pilát mycelia (PBMP). (A) Elution curve of crude PBMP on DEAE Cellulose-52 chromatography column in which F1 was the fraction obtained by elution with deionized water, F2 was the fraction eluted with 0.1 mol/L NaCl solution, F3 was the fraction eluted with 0.3 mol/L NaCl solution, (B) Elution curve of PBMP fraction 1 ($PBMP_1$) on Sephadex G-100 chromatography column, and (C) Relative molecular weight of $PBMP_1$.

3. Results and discussions

3.1. Isolation, purification and components of $PBMP_1$

This study showed that PBMP was performed by DEAE-cellulose chromatography, and three independent elution peaks (called the fraction (F)1 was the fraction obtained by elution with deionized water, F2 was the fraction eluted with 0.1 mol/L NaCl solution, F3 was the fraction eluted with 0.3 mol/L NaCl solution) were obtained (Fig. 1A). Subsequently, F1 was purified using Sephadex G-100, which also yielded a single elution peak (Fig. 1 B), $PBMP_1$, the yield was 32.57 % compared to PBMP (Table 1). This also implied that $PBMP_1$ was the primary PBMP. Moreover, this study indicated that the total carbohydrate, uronic acid, Fuc, Glc, and Gal contents of $PBMP_1$ were 99.49, 2.1, 1.00, 24.54, and 0.29 % (Table 1), respectively. In particular, the total carbohydrates were 99.49 %, indicating no non-sugar components such as lipid chains and peptide chains in $PBMP_1$. Moreover, three monosaccharide fractions with varying Fuc, Glc, and Gal suggested that $PBMP_1$ is a heteropolysaccharide. Additionally, the glucuronic acid content was determined to be 2.1 %, confirming that $PBMP_1$ is an acidic heteropolysaccharide. Well-known, typically fungal polysaccharides such as homo- and hetero-polysaccharides which may be composed of monosaccharides such as Glc, Gal, arabinose, fructose, hexose, Man, and rhamnose [2,10,24]. These water-soluble polysaccharides were the typical extraction (hot water extraction and ethanol precipitation) approaches with partial loss due to variations in conditions, thus accumulating bio-waste deposits and losing the bioactive polysaccharides [25]. However, it was noted that modified by strain mutations resulting in altered monosaccharide composition and MW distribution in polysaccharides improved antioxidant activity, contained significantly less D-Glc and more L-rhamnose, and included 61 % 1.5 kDa small molecule polysaccharide fragments and low uronic acid content [26]. This also signifies that the antioxidant activity of polysaccharides decreases with increasing structural complexity and molecular weight of polysaccharides, especially uronic acid content [27]. However, this study indicates that $PBMP_1$ contains additional Fuc, apart from the typical hetero-polysaccharide composition of Glc and Gal, compared to previous studies on *Phellinus* polysaccharides [11,12,25].

In addition, this study exhibited a negative Bradford assay response, and the lack of absorption at 280 nm indicated that the $PBMP_1$ was protein-free. It has also been reported the modification of PBMP by carboxymethylation to equip it with (1 → 3)-β-glucan with a (1 → 6)-linkage (MW 1.16×10^6 Da) and enhance its biological activity [28]. However, the non-sugar component on the side chain of $PBMP_1$ (as complex polysaccharides) identified in this study resembles glycosides, which will be described in the structural analysis. This study's result was echoed in the HPGPC analysis (Fig. 1C); namely, the relative MW of $PBMP_1$ (retention time of 6.821 min) was estimated to have an MW of approximately 2.95×10^3 kDa through Shodex standard P-82 pullulan HPGPC elution (Table 1). T. Li et al. [9] reported that in liquid-state fermentation extracts of MP, the MW belongs to the macromolecule (2.01×10^7 Da); in contrast, solid-state fermentation contributes to the fractionation of low-MW polysaccharides. It has been reported that PBMP with large MW typically exhibited more excellent anticancer activity [27,29]. Notably, it has been reported that the mycelial polysaccharide of *P. igniarius* at a concentration of 400 μg/mL and with a molecular weight of 34.1 kD demonstrated remarkable growth inhibition (60–70 %) in HePG2 and SW480 cell lines [12]. The enhanced anti-tumor activity of polysaccharides with large molecular size and favorable water solubility, specifically attributed to the presence of carboxylate groups, has also been documented [30]. Concretely, the biological activities of polysaccharides are influenced by various structural characteristics, such as MW, the composition of monosaccharides, glycosidic bond type, chain conformation, and degree of branching [12,27]. However, it is widely acknowledged that the antioxidant activity of polysaccharides tends to decline as their structural complexity and MW increase, particularly concerning uronic acid content [27]. Moreover, GC analysis showed $PBMP_1$ was composed of glycerol (retention time (RT) of 7.749 min), erythritol (RT of 15.544 min), and Glc (RT of 26.280 min) (Fig. 2) with relative molar ratios of about 1.00: 24.54: 0.29. The compositional differences above have been reported to be potentially affected by different culture conditions (strains, fermentation, extraction, isolation approaches, etc.) on the monosaccharide compositions [8–11].

3.2. Functional group composition of $PBMP_1$

This study was conducted to characterize $PBMP_1$ using FT-IR spectroscopy (Fig. 3), which indicated that a strong and broad absorption peak at 3393.21 cm^{-1} was attributed to hydroxyl stretching vibrations [7,19]. In addition, it was observed that the absorbance peak at 2930.66 cm^{-1} for C–H stretching vibrations [8,30] and the three absorption peaks spanning 1000 to 1200 cm^{-1} for coupled C–O and C–C stretching and C–OH bending vibrations were observed [8,20,25,31]. The band at 1654.75 cm^{-1} was due to the bound water [32]. The band at 847.92 cm^{-1} was attributed to α-type glycosidic linkages in the polysaccharide [33]. Additionally, the bands at 930.19 and 761.82 cm^{-1} were characteristic of D-pyranose-glucan [6]. The infrared spectrum indicated $PBMP_1$ contained α-glycosidic linkages [34]. Therefore, the results above indicate that $PBMP_1$ purified in this study was equipped with the structure of polysaccharides.

Table 1
Yields, protein, uronic acid, monosaccharide contents, and molecular weight for *Phellinus baumii* Pilát mycelial polysaccharide (PBMP) fraction 1 ($PBMP_1$).

Sample	Yield[a]	Carbohydrate (%)	Protein (%)	Uronic acid (%)	Fucose (%)	Glucose (%)	Galactose (%)	Molecular weight (kDa)
$PBMP_1$	32.57	99.49	ND	2.01	1.00	24.54	0.29	2.95×10^3

ND represents not detected.
[a] Calculated as the weight ratio of $PBMP_1$/PBMP (crude polysaccharide).

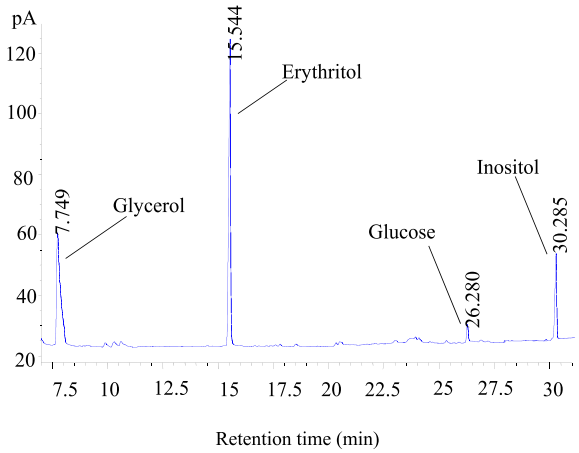

Fig. 2. Gas chromatography-mass spectrometer (GC-MS) spectrum of monosaccharide composition of polysaccharide fraction 1 from *Phellinus baumii* Pilát mycelia ($PBMP_1$).

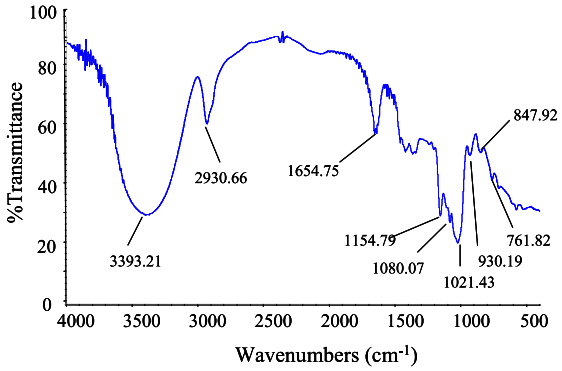

Fig. 3. Fourier-transform infrared (FT-IR) spectra of polysaccharide fraction 1 from *Phellinus baumii* Pilát mycelia ($PBMP_1$).

3.3. Periodic acid oxidation, smith degradation, and GC spectra of $PBMP_1$

The GC–MS analysis results were consistent with those from Periodate oxidation and Smith degradation, indicating that 2, 3, 6-Me_3-Glc (1, 4-linked Glc) are major components of the backbone structure with 3 side chains. They were 1→6-linked-Gal, (1 → 3, 4)-linked-Glc, and 1 → 4, 6-linked-Glc, respectively, while these findings appear similar to those reported by Yuan et al. [12]. Notably, the linear repetitive backbone consists of Glc*p*, Gal*p*, and Man*p* [1]. The terminal non-reducing Fuc and Glc residues were also observed. Therefore, it was concluded that the structure of $PBMP_1$ was complex, and the results demonstrated that $PBMP_1$ had a backbone chain composed of 1, 4-linked Glc residues with three 1, 3- and 1, 6-branched chains [35].

This study showed that $PBMP_1$ was derivatized by periodate oxidation-Smith degradation reaction and analyzed by GC utilizing the saccharonitrile acetate method, while from GC spectra, much glycerol and erythritol were found (Fig. 3). This study revealed that there were many 1→, 1 → 2, 1 → 6, 1 → 2, 6, 1 → 4 and 1 → 4, 6 linkages in $PBMP_1$. Moreover, the content of erythritol was much more than that of glycerol and monosaccharide in $PBMP_1$. This also implied the major existence of monosaccharides with 1 → 4 or 1 → 4, 6 linked glycosidic bonds. The presence of Glc revealed that some residues of Glc were 1 → 3 or 1 → 2, 3 or 1 → 2, 4 or 1 → 3, 4 or 1 → 3, 6 or 1 → 2, 3, 6 or 1 → 2, 4, 6 or 1 → 3, 4, 6 linked glycosidic bonds that cannot be oxidized, respectively.

Moreover, the physicochemical and structural properties of PBMPs, specifically the MW, monosaccharide composition, glycosidic bond, and chain conformation, are well-known and related to the activities performed by their pharmacologists [12]. In this study, the Methylation analysis of $PBMP_1$ by GC–MS showed the presence of six main components, and their main fragments (m/e), molar ratio, and linkage mode were displayed in Table 2. These components were 2, 3, 4-Me_3-Fuc, 2, 3, 4, 6- Me_4-Glc, 2, 3, 6-Me_3-Glc, 2, 3, 4-Me_3-Gal, 2, 6-Me_2-Glc, and 2, 3-Me_2-Glc in molar ratios of 8:7:17:6:1:4. These results were similar to those reported for SSEPS2 (Me-Man*p*) by Cheng et al. [8] and PBF4 ((1 → 2)-L-Fuc*p*) by Ge et al. [36]. However, the variations still depended on the specific strains and culture conditions used during the development process. However, monosaccharides with structures such as 3-O-Me-Glc have also been reported to have immunosuppressive and anti-inflammatory activities [37].

3.4. NMR spectra of $PBMP_1$

The ^{1}H NMR results of PBMP1 in this study showed that the peak signal at δ 4.699 ppm was the heavy water peak (Fig. 4A); the resonance peak region of δ 4.70–5.50 ppm for the anomeric hydrogen was dominated by two prominent anomeric hydrogen resonance peaks signals at δ 4.86 and 5.28 ppm (α-configurations anomeric signals) [7,38], respectively. This implies that the $PBMP_1$ glycosidic bond constitution was predominantly of the α-type. Notably, at δ 5.28 ppm, the peak tip of the isohead hydrogen was distinct, and the bottom was wider. It was hypothesized that the possibility was due to the non-separated or stacked hetero-head hydrogen of different sugar residues. In addition, the spectral peaks between δ 3.20 and 4.40 ppm were generated by stacking proton signals on C_2 to C_6 [39], which were difficult to resolve. A similar result, in that the range corresponds to sugar ring protons, has been reported by Cheng et al. [8] and Sun et al. [11]. However, a weak peak at δ 1.2 ppm was probably the methyl doublet of Fuc H-6 (J5, 6 = 7.10 Hz). The amino, sulfate, and other groups created the peaks at 0.60–3.20, 5.50–6.40, and 6.60–8.40 ppm [39,40].

Regarding the ^{13}C NMR results, this study showed four primary carbon signals at δ 97.75–102.00 ppm (Fig. 4B), while the signals were 98.99, 99.98, 100.25, and 100.56 ppm, respectively. This similarly resulted as described above, which means that the $PBMP_1$ glycosidic bond was conformed to the α-type [8]. However, there was a resonance peak for Fuc C_6 at δ 22.76 ppm and the carbon signal for the 6-position substitution at 60.50–65.46 ppm [35]. In addition, the other oxygen-linked carbon signals in the sugar ring were at 70.54–73.55 ppm. Remarkably, there is no resonance signal at δ 82–88 ppm on the ^{13}C NMR spectrum, proving that all the sugar residues in the $PBMP_1$ in this study are glycosides of pyranose structures [8,35]. Therefore, these results of NMR spectra were consistent with that of FTIR spectra in section 3.3. Thus, these results indicate different variations in the composition, structure, and bioactivity of the PBMP by various strains [11,27], particularly polysaccharides with monosaccharide units composed of glycosidic linkages [35,41]. Despite these constructions that have been clarified involving the biological activity and health-promoting benefits of PBMP, both series systems and extensive toxicology and safety evaluations will be necessary [1,29]. Simultaneously, it is imperative to consider the growth of industries dedicated to ensuring water, energy, and food security [42,43]. This also contributes to realizing the Sustainable Development Goals (SDGs) proposed by the United Nations and better realizing a biologically based circular economy [42–44].

4. Conclusions

This study revealed that $PBMP_1$ comprises Fuc, Glc, and Gal. The structural analysis using FTIR and NMR techniques confirmed its polysaccharide nature, exhibiting characteristic signals corresponding to the pyranose ring. Specifically, $PBMP_1$ is a hetero-polysaccharide with an α-glycosidic linkage and highly branched 1,3,4-Glc and 1,4,6-Glc branches with Fuc as the non-reducing terminus. Moreover, the limitations of this study lie in the utilization of separation and purification techniques at a laboratory scale, which may give rise to concerns regarding residual organic solvents. These potential issues could potentially impact the bioactivity of medicinal metabolites and impede the practical application of these products. Therefore, it is imperative to investigate environmentally friendly extraction and separation techniques, such as membrane separation and ultrafiltration, which are crucial for pilot and industrial applications. It also implied that using our previously developed optimized culture conditions facilitated the mass production of $PBMP_1$. The advancement of extraction and purification techniques was anticipated to yield a groundbreaking approach for highlighting the health-promoting potential of PBMP1 and harnessing it to its most total advantage.

Data availability statement

Data included in the article, and all the data supporting this study's findings are available from the corresponding author upon reasonable request.

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Table 2
Gas chromatography-mass spectrometer (GC-MS) analysis of methylated sugar residues components of polysaccharide fraction 1 from *Phellinus baumii* Pilát mycelial ($PBMP_1$).

Methylated sugars	Main fragments (m/e)	Molar ratio	Type of linkage
2,3,4 - Me_3 - Fuc	43, 59, 71, 87, 101, 117, 131, 143, 151, 175, 233	8	Fuc-(1→
2,3,4,6- Me_4 - Glc	43, 59, 71, 87, 101, 117, 129, 143, 161, 189, 233	7	Glc-(1→
2,3,6 - Me_3 - Glc	43, 71, 85, 101, 117, 131, 157, 171, 233	17	→4)-Glc-(1→
2,3,4 - Me_3 - Gal	43, 59, 71, 87, 101, 117, 129, 143, 151, 189	6	→6)-Gal-(1→
2,6-Me_2-Glc	43, 59, 71, 87, 101, 117, 129, 143, 161, 189, 233	1	→3,4)-Glc-(1→
2,3-Me_2-Glc	43, 57, 71, 85, 101, 117, 129, 142, 159, 187, 201, 261	4	→4,6)-Glc-(1→

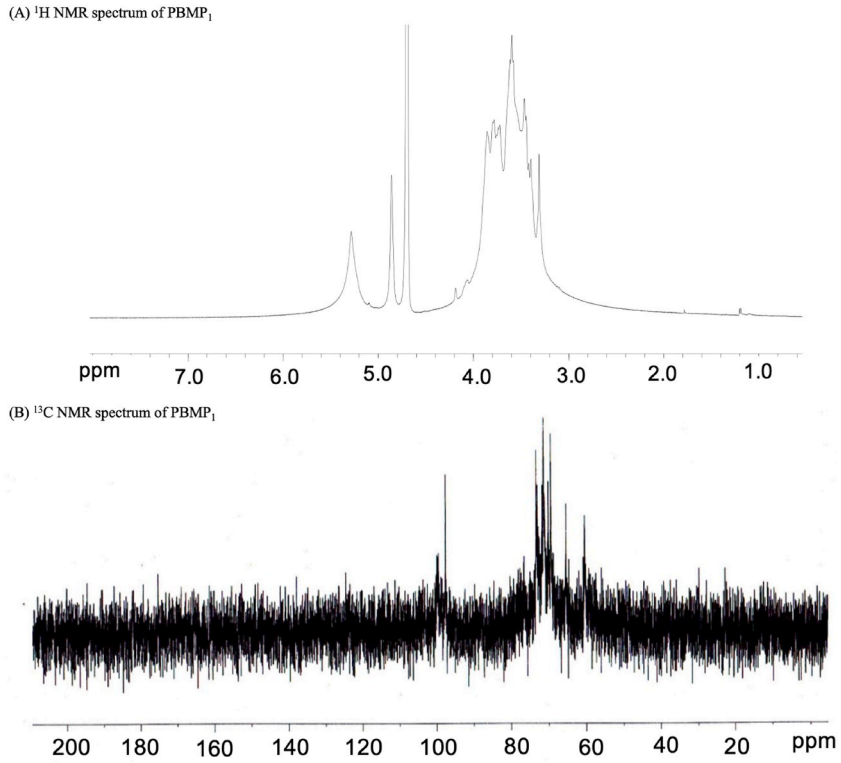

Fig. 4. (A)^{1}H Nuclear magnetic resonance (NMR) and (B)^{13}C NMR spectrum for polysaccharide fraction 1 from *Phellinus baumii Pilát* mycelia (PBMP$_1$).

CRediT authorship contribution statement

JianGuang Luo: Writing – original draft, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Conceptualization. **JunYan Wu:** Validation, Methodology, Formal analysis, Data curation, Conceptualization. **YiNi Xu:** Validation, Methodology, Investigation, Formal analysis. **Hu Shao:** Writing – review & editing, Resources, Project administration, Investigation. **Ping-Hsiu Huang:** Writing – review & editing, Writing – original draft, Visualization, Software.

Declaration of generative AI and AI-assisted technologies in the writing process

No, this article does not use AI and AI-assisted technologies in writing.

Declaration of competing interest

The manuscript remains unpublished and has not been submitted to or considered for publication in any other academic journal. The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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