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Structural characterization of an immunoenhancing heteroglycan of a hybrid mushroom (*pfls1h*) of *Pleurotus florida* and *Lentinus squarrosulus* (Mont.) Singer

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ABSTRACT

An immunostimulating water-soluble heteroglycan (PS-II) was isolated from an aqueous extract of the fruit bodies of a hybrid mushroom, *pfls1h* produced by intergeneric protoplast fusion between *Pleurotus florida* and *Lentinus squarrosulus* (Mont.) Singer. Structural characterization of PS-II was carried out using sugar analysis, methylation experiment, periodate oxidation and 1D/2D NMR studies. Sugar analysis indicated the presence of glucose, mannose and galactose in a molar ratio of 1:1:2. Methylation analysis revealed that PS-II was composed of (1→6)- and (1→2,4,6)-α-D-galactopyranosyl, terminal β-D-mannopyranosyl and terminal β-D-glucopyranosyl residues in a relative proportion of approximately 1:1:1:1. On the basis of chemical analysis and NMR studies, the structure of the repeating unit of the heteroglycan was established to consist of a backbone chain of two (1→6)-α-D-galactopyranosyl residues, one of which is branched at *O*-2 with terminal β-D-Man*p* and at *O*-4 with terminal β-D-Glc*p*. This heteroglycan (PS-II) showed in vitro splenocyte, thymocyte and macrophage activations.

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1. Introduction

Mushrooms which are used as foodstuff for their taste and nutritional value from ancient times, also exhibit important medicinal properties.[1] During the last few decades polysaccharides isolated from different mushrooms have become an interesting topic for the researcher in the field of biochemistry and pharmacology for wide application of their anti-tumor, immunostimulating, and antioxidant properties.[2–4] A series of polysaccharides like (1→6)-α-D-glucan,[5] (1→3)-, (1→6)-α,β-D-glucan,[6] heteroglycan consisting of glucose, mannose and galactose[7] and (1→3)-,(1→6)-β-D-glucan[8] have been isolated from water soluble, insoluble and NaCl soluble fractions of an edible mushroom *Pleurotus florida* and reported earlier. Immunostimulating heteroglycans[9] consisting of galactose, glucose and fucose and (1→3)-, (1→6)-β-D-glucan[10] were also reported from the aqueous and alkaline extracts of another edible mushroom *Lentinus squarrosulus* (Mont.) Singer, respectively. Twelve new hybrid strains were developed through intergeneric protoplast fusion[11] between *Pleurotus florida* and *Lentinus squarrosulus* (Mont.) Singer by adopting the method reported earlier,[12] out of which six strains that are, *pfls 1h, pfls 1j, pfls 1k, pfls 1m, pfls 1n, and pfls 1p* were found to produce fruit bodies. Aqueous extract of fruit bodies of the hybrid mushroom strain, *pfls 1h* yielded two polysaccharides, glucan (PS-I) and heteroglycan (PS-II). Detailed structural characterization and immunostimulating activities of the (1→3)-, (1→6)-β-D-glucan (PS-I) obtained from the hybrid mushroom, *pfls1h* were reported recently.[11] In the present investigation structural characterization and immunoenhancing activities of the heteroglycan (PS-II) were carried out and reported herein.

2. Results and discussion

2.1. Purification and chemical analysis of PS-II

The fresh fruit bodies (500 g) of the hybrid mushroom *pfls1h* were washed with distilled water followed by extraction with hot water, precipitation in alcohol, dialysis, centrifugation and freeze drying to yield crude polysaccharide (1.9 g). Two fractions of purified polysaccharides were obtained after fractionating water soluble crude polysaccharide (30 mg) through a Sepharose 6B column. Two fractions, fraction I and fraction II were collected and freeze dried, yielding purified polysaccharide of 9 mg PS-I and 11 mg PS-II, respectively. PS-II showed a specific rotation of $[\alpha]_D^{31}$ +45.8 (*c* 0.1, water). The molecular

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weight of PS-II was estimated as $\sim 1.48 \times 10^5$ Da from a calibration curve prepared using standard dextran. GC analysis of the alditol acetates of the acid-hydrolyzed PS-II revealed the presence of glucose, mannose and galactose in molar ratio of 1:1:2. Determination of the absolute configuration of the monosaccharides showed that all the three monosaccharides were present in the D configuration. GC–MS analysis of partially methylated alditol acetates revealed the presence of 1,2,4,5,6-tetra-*O*-acetyl-3-*O*-methyl-galactitol, 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-galactitol, 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-mannitol, 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-glucitol and thus, PS-II was deduced to consist of (1→2,4,6)-, (1→6)-α-D-galactopyranosyl, terminal β-D-mannopyranosyl and terminal β-D-glucopyranosyl residues in a relative proportion of approximately 1:1:1:1. GC analysis of alditol acetates of the periodate-oxidized[13,14], $NaBH_4$-reduced PS-II was found to contain D-galactose only. GC-MS analysis of periodate-oxidized, reduced, and methylated PS-II showed that only the monomethyl sugar residue, 1,2,4,5,6-penta-*O*-acetyl-3-*O*-methyl-D-galactitol was retained. This result indicates that (1→6)-linked D-galactopyranosyl and both the terminal D-glucopyranosyl and D-mannopyranosyl residues were consumed during oxidation which further confirmed the mode of linkages present in PS-II.

2.2. Structural analysis of PS-II

The ^{1}H NMR spectrum (Fig. 1) of PS-II at 30 °C showed the presence of four signals in the anomeric region at δ 5.13, 4.99, 4.80, and 4.52 in a ratio of nearly 1:1:1:1. The four anomeric proton signals correspond to four sugar residues, designated as **A**, **B**, **C** and **D** according to their decreasing proton chemical shift values. In both ^{13}C (Fig. 2a) and DEPT-135 (Fig. 2b) spectra four signals were found in the anomeric region at δ 102.8, 101.7, 98.7, and 98.1. From the HSQC spectrum (Fig. 3), the anomeric proton signal of residue **A** at δ 5.13 was correlated to the anomeric carbon signal at δ 98.7 and the anomeric proton signal of residue **B** at δ 4.99 was correlated to the anomeric carbon signals at δ 98.1. Again, the anomeric proton signal at δ 4.80 was correlated to the anomeric carbon signal at δ 101.7 corresponding to the anomeric carbon of residue **C** and the anomeric proton signal of residue **D** at δ 4.52 was correlated to the anomeric carbon signal at δ 102. All the ^{1}H and ^{13}C signals (Table 1) were assigned from DQF-COSY, TOCSY, and HSQC experiments. The proton coupling constants were measured from DQF-COSY experiment.

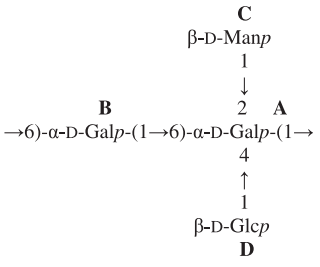
The galacto configuration of residues **A** and **B** was assigned from the large $J_{H\text{-}2,H\text{-}3}$ coupling constant (~8 Hz) and relatively small $J_{H\text{-}3,H\text{-}4}$ coupling constant (0–3 Hz). The α-configuration of both **A** and **B** residues was assigned from $J_{H\text{-}1,H\text{-}2}$ coupling constant (~3 Hz) and $J_{C\text{-}1,H\text{-}1}$ of (~170 Hz). The downfield shift of C-2 (δ 77.0), C-4 (δ 76.1), and C-6 (δ 66.7) with respect to standard values of methyl glycosides[15,16] indicate that residue **A** was a (1→2,4,6)-D-galactopyranosyl residue. The downfield shift of C-6 (δ 67.0) with respect to the standard value of methyl glycosides[15,16] indicate that residue **B** was a (1→6)-linked D-galactopyranosyl residue. The downfield shift and downward displacement of the C-6 signals (Fig. 2b) corresponded to CH_2 carbons of residues **A** and **B** in DEPT-135 spectrum further confirmed that these residues were linked at C-6.

Residue **C** was assigned to the terminal D-mannopyranosyl moiety. The manno configuration of residue **C** was supported from large coupling constants of $J_{H\text{-}3,H\text{-}4}$ (~7 Hz) and $J_{H\text{-}4,H\text{-}5}$ (~9 Hz). The anomeric proton and carbon signals at δ 4.80 and 101.7 indicated that D-mannose was β-linked. The carbon chemical shifts of residue **C** from C-1 to C-6 nearly corresponded to the standard values of methyl glycoside of β-D-mannose indicating **C** was terminal β-D-Man*p*.

The large $J_{H\text{-}2,H\text{-}3}$ and $J_{H\text{-}3,H\text{-}4}$ coupling constant values (~10 Hz) of residue **D** confirmed its D-glucopyranosyl configuration. The residue **D** with anomeric proton and carbon chemical shifts at δ 4.52 and 102.7, respectively and the coupling constant values $J_{H\text{-}1,H\text{-}2}$ (~8 Hz), $J_{C\text{-}1,H\text{-}1}$ (~160–161 Hz) confirmed that the residue was present in β-configuration. The carbon chemical shifts of residue **D** from C-1 to C-6 corresponded nearly to the standard values of methyl glycoside of β-D-glucose indicating **D** was terminal β-D-Glc*p*.

The sequence of glycosyl residues was established from NOESY as well as ROESY (not shown) experiments. In NOESY experiment (Fig. 4, Table 2), the inter-residual contacts from **A**H-1/**B**H-6a, **B**H-6b; **A**H-1/**C**H-1; **B**H-1/**A**H-6a, **A**H-6b; **C**H-1/**A**H-1; **C**H-1/**A**H-2; and **D**H-1/**A**H-4 along with other intra-residual contacts were observed.

A long range HMBC experiment was carried out to confirm the connectivities obtained from NOESY experiment. Inter-residual cross-peaks **A**H-1/**B**C-6; **A**C-1/**B**H-6a, **B**H-6b; **B**H-1/**A**C-6; **B**C-1/**A**H-6a, **A**H-6b; **C**H-1/**A**C-2; **C**C-1/**A**H-2; and **D**H-1/**A**C-4; **D**C-1/**A**H-4 along with some intra-residual cross peaks were also observed (Fig. 5 and Table 3). Thus, the HMBC and NOESY connectivities clearly supported the presence of a tetrasaccharide repeating unit in the heteroglycan (PS-II) isolated from a hybrid mushroom, *pfls1h* of *Pleurotus florida* and *Lentinus squarrosulus* (Mont.) Singer and established as:



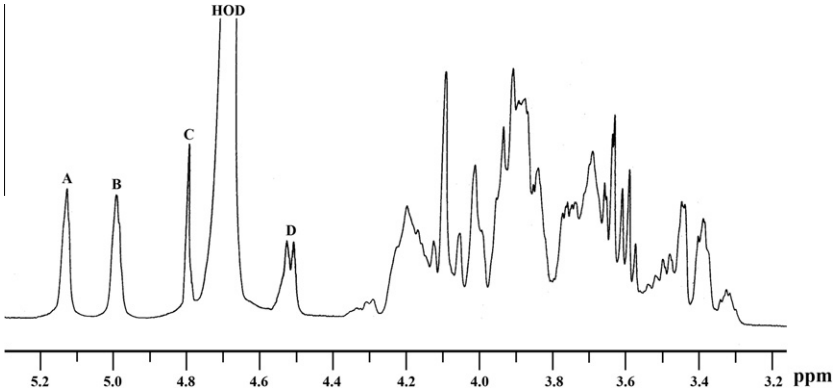


Figure 1. ^{1}H NMR spectrum (500 MHz, D_2O, 30 °C) of PS-II of a hybrid mushroom (*pfls1h*) of *Pleurotus florida* and *Lentinus squarrosulus* (Mont.) Singer.

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